

AD _____

Award Number: DAMD17-97-1-7075

TITLE: Cloning and Biochemical Analysis of the
Ataxia-Telangiectasia (A-T) Gene in Xenopus Laevis

PRINCIPAL INVESTIGATOR: Carmel E. Hensey, Ph.D.

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10032

REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 4

20010216 105

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)**2. REPORT DATE**
September 1999**3. REPORT TYPE AND DATES COVERED**
Annual (1 Sep 98 - 31 Aug 99)**4. TITLE AND SUBTITLE**Cloning and Biochemical Analysis of the Ataxia-Telangiectasia (A-T) Gene in *Xenopus Laevis***5. FUNDING NUMBERS**
DAMD17-97-1-7075**6. AUTHOR(S)**

Carmel E. Hensey, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)Columbia University
New York, New York 10032**E-MAIL:**
cehl18@columbia.edu**8. PERFORMING ORGANIZATION REPORT NUMBER****9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES****12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

ATM, the gene product mutated in Ataxia Telangiectasia (A-T) encodes a 350-kDa protein involved in the regulation of several cellular responses to DNA breaks. We used a degenerate PCR-based strategy to isolate a partial clone of X-ATM, the *Xenopus* homologue of human ATM. Sequence analysis confirmed that the clone was most closely related to human ATM. *Xenopus* ATM protein (X-ATM) is 85% identical to human ATM within the kinase domain and 71% identical over the carboxyl-terminal half of the protein. Polyclonal antibodies raised against recombinant X-ATM are highly specific for the ATM protein and recognize a single polypeptide of 370-kDa in oocytes, embryos, egg extracts and a *Xenopus* cell line. We found that X-ATM was expressed maternally in eggs and as early as stage II pre-vitellogenic oocytes, and the protein and mRNA were present at relatively constant levels throughout development. Subcellular fractionation showed that the protein was nuclear in both the female and male germlines. The level of X-ATM protein did not change throughout the meiotic divisions or the synchronous mitotic cycles of cleavage stage embryos. In addition, we did not observe any change in the level or mobility of X-ATM protein following γ -irradiation of embryos. Finally, we also demonstrated that X-ATM was present in a high molecular weight complex of approximately 500 kDa containing the X-ATM protein and other, as yet unidentified component(s).

14. SUBJECT TERMS

Breast Cancer

15. NUMBER OF PAGES

43

16. PRICE CODE**17. SECURITY CLASSIFICATION OF REPORT**

Unclassified

18. SECURITY CLASSIFICATION OF THIS PAGE

Unclassified

19. SECURITY CLASSIFICATION OF ABSTRACT

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

CH X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

CH N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

CH X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

CH X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

CH X In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Carmel Hensley Sept 30th 99
PI - Signature Date

Carmel Hensey

Cloning and biochemical analysis of the Ataxia Telangiectasia (A-T) gene in *Xenopus laevis*.

Annual Report, September 1999

TABLE OF CONTENTS

SECTION	PAGE
Introduction	4
Body	4
Appendices	13

Introduction

Ataxia Telangiectasia (A-T) is an autosomal recessive disease which displays a complex phenotype (Boder & Sedgwick, 1970; Shiloh, 1998). Patients exhibit a progressive cerebellar ataxia, in addition to severe immune deficiencies, gonadal atrophy, telangiectases, increased risk for cancer, particularly lymphomas, and radiation sensitivity. Additionally, carriers are suspected to be prone to other cancers including breast cancer (Athma *et al.*, 1996; Chen *et al.*, 1998; Stankovic *et al.*, 1999; Swift *et al.*, 1990; Yuille & Coignet, 1998). *Xenopus laevis* is a powerful model system for both biochemical studies of cell cycle and checkpoint regulation as well as for developmental studies. We decided to use *Xenopus* as a novel model system to study both the biochemical role of ATM and its function during development.

Body

This report describes the progress made in studying ATM for the award period September 1st 1998-August 31st 1999.

Xenopus laevis ATM (X-ATM)

Using library screening, ATM specific library construction, and 5'RACE (Figure 1A) sequence corresponding to approximately 50 % of the predicted *Xenopus* ORF was obtained (Figure 1A). An alignment of the carboxyl-terminal half of X-ATM with human ATM is shown in figure 1B. ATM is highly conserved between *Xenopus* and human with an overall identity of 71% in the region we have sequenced. Within the kinase domain the identity is 85% while the region upstream of the kinase domain (amino acids 1 to 1100 of X-ATM) shows 66% identity to the human protein.

Subcellular localization of the ATM protein in the germline.

We examined whether the protein was already present in the oocyte as opposed to being synthesized during meiotic maturation as has been described for some proteins (Sagata *et al.*, 1988). *Xenopus* oocytes were manually dissected from pieces of ovary and protein extracts were made at the stages of oogenesis indicated (Dumont, 1972). X-ATM is already present in previtellogenic oocytes (stage II) and persists throughout oogenesis (Figure 2A). Although the concentration of X-ATM per mass of tissue does not vary significantly throughout oogenesis, the mass per oocyte increases dramatically since the volume of the oocytes increases by a factor of 10^3 between stages I-II and stage VI.

Stage VI *Xenopus* oocytes contain a large (200 μ m diameter) nucleus, known as the germinal vesicle (GV), which can be used to store nuclear proteins important for later development (Dreyer & Hausen, 1983). The GV can be isolated surgically from its surrounding cytoplasm,

allowing the preparation of pure cytoplasmic and nuclear fractions from oocytes (Ford & Gurdon, 1977). We demonstrate that X-ATM is entirely nuclear in stage VI oocytes (Figure 2B). Moreover, using either demembranated sperm nuclei, or a nuclear extract prepared from these nuclei following their incubation in extracts (Walter *et al.*, 1998), we show that X-ATM is also nuclear in the male germline (Figure 2B).

Expression of X-ATM protein throughout the meiotic and mitotic cell cycles.

In *Xenopus* oocytes the highly synchronous meiotic cell cycle is easily studied in vitro, where progesterone induced oocyte maturation culminates in the completion of meiosis I and the progression to metaphase of meiosis II. To determine whether X-ATM protein levels fluctuated during the meiotic cell cycle, we performed Western blots on protein samples from oocytes synchronously undergoing meiosis. X-ATM protein levels remained constant throughout meiosis (Figure 3A). In this experiment, prophase-arrested oocytes were induced to undergo meiotic maturation in vitro by progesterone. Nuclear envelope breakdown took place at 3.5 hrs, meiosis I occurred at 6 hrs and the oocytes were arrested in metaphase of meiosis II by 9 hrs.

We also analyzed X-ATM levels throughout the mitotic cell cycle where cell cycle oscillations were assessed by following cdc2/cyclinB activity. The naturally synchronous cell divisions of the early embryo allow the preparation of cell free extracts that undergo cell cycle oscillations in vitro. Using such extracts we examined X-ATM protein levels throughout these highly synchronized cell cycles. ATM is expressed throughout the cell cycle and its levels remain constant over the course of two cell cycles (Figure 3B). Additionally, there was no change in the electrophoretic mobility of X-ATM.

X-ATM protein in γ -irradiated eggs

In *Xenopus*, irradiation of cleavage stage embryos leads to massive and synchronous apoptosis at the onset of gastrulation (St. 10.5) (Anderson *et al.*, 1997; Hensey & Gautier, 1997). To assess whether this response correlated with changes in the amounts or the electrophoretic mobility of X-ATM, we compare the levels of X-ATM in protein samples from untreated and γ -irradiated embryos between stage 8 and 10.5. X-ATM protein levels were similar in control and irradiated embryos. In addition no change in the mobility of the protein was detected (Figure 4).

X-ATM is present in a protein complex in *Xenopus* extracts.

The carboxyl-terminal kinase domain of ATM represents a small portion of this large protein. Little is known about the function of the domain(s) outside the kinase region but support for its importance comes from both genetic analysis of ATM mutants (Concannon & Gatti, 1997) as well as the finding that a putative leucine zipper region of ATM behaved as a transdominant negative

(Morgan *et al.*, 1997). A central proline rich domain has already been shown to interact with the c-abl SH3 domain (Shafman *et al.*, 1997), and the remainder of the protein is suspected of playing regulatory functions by interacting with other proteins. To determine whether X-ATM might be associated with other proteins in *Xenopus* extracts we analyzed extracts by native gel electrophoresis. We detected a native, high molecular weight X-ATM-containing complex with an estimated size of 500-kDa (Figure 5).

REFERENCES

- Anderson, J.A., Lewellyn, A.L. & Maller, J.L. (1997). *Mol Biol Cell*, **8**, 1195-206.
- Athma, P., Rappaport, R. & Swift, M. (1996). *Cancer Genet Cytogenet*, **92**, 130-4.
- Boder, E. & Sedgwick, R.P. (1970). *Psychiatr Neurol Med Psychol Beih*, **14**, 8-16.
- Brown, A.L., Lee, C.H., Schwarz, J.K., Mitiku, N., Piwnica-Worms, H. & Chung, J.H. (1999). *Proc Natl Acad Sci U S A*, **96**, 3745-3750.
- Chen, J., Birkholtz, G.G., Lindblom, P., Rubio, C. & Lindblom, A. (1998). *Cancer Res*, **58**, 1376-9.
- Concannon, P. & Gatti, R.A. (1997). *Hum Mutat*, **10**, 100-7.
- Dreyer, C. & Hausen, P. (1983). *Dev Biol*, **100**, 412-25.
- Dumont, J.N. (1972). *J Morphol*, **136**, 153-79.
- Ford, C.C. & Gurdon, J.B. (1977). *J Embryol Exp Morphol*, **37**, 203-9.
- Hensey, C. & Gautier, J. (1997). *Mech Dev*, **69**, 183-95.
- Morgan, S.E., Lovly, C., Pandita, T.K., Shiloh, Y. & Kastan, M.B. (1997). *Mol Cell Biol*, **17**, 2020-9.
- Sagata, N., Oskarsson, M., Copeland, T., Brumbaugh, J. & Vande Woude, G.F. (1988). *Nature*, **335**, 519-25.
- Shafman, T., Khanna, K.K., Kedar, P., Spring, K., Kozlov, S., Yen, T., Hobson, K., Gatei, M., Zhang, N., Shiloh, Y. (1998). *Biochim Biophys Acta*, **1378**, R11-8.
- Stankovic, T., Weber, P., Stewart, G., Bedenham, T., Murray, J., Byrd, P.J., Moss, P.A. & Taylor, A.M. (1999). *Lancet*, **353**, 26-9.
- Swift, M., Chase, C.L. & Morrell, D. (1990). *Cancer Genet Cytogenet*, **46**, 21-7.
- Walter, J., Sun, L. & Newport, J. (1998). *Mol Cell*, **1**, 519-29.
- Yuille, M.A. & Coignet, L.J. (1998). *Recent Results Cancer Res*, **154**, 156-73.

Figure 1A

ATM domains and cloning strategy. The human ATM protein is represented by a thick black line, specific domains are indicated. X-ATM clones are indicated by arrows. T7/TBH-XTC denotes the in vitro translated fragment of X-ATM used to screen sera from rabbits A and B. PQE60/TBH6H was used in antigen production and part of this region was used in RNase protection assays (construct T7/TBH-XTC)

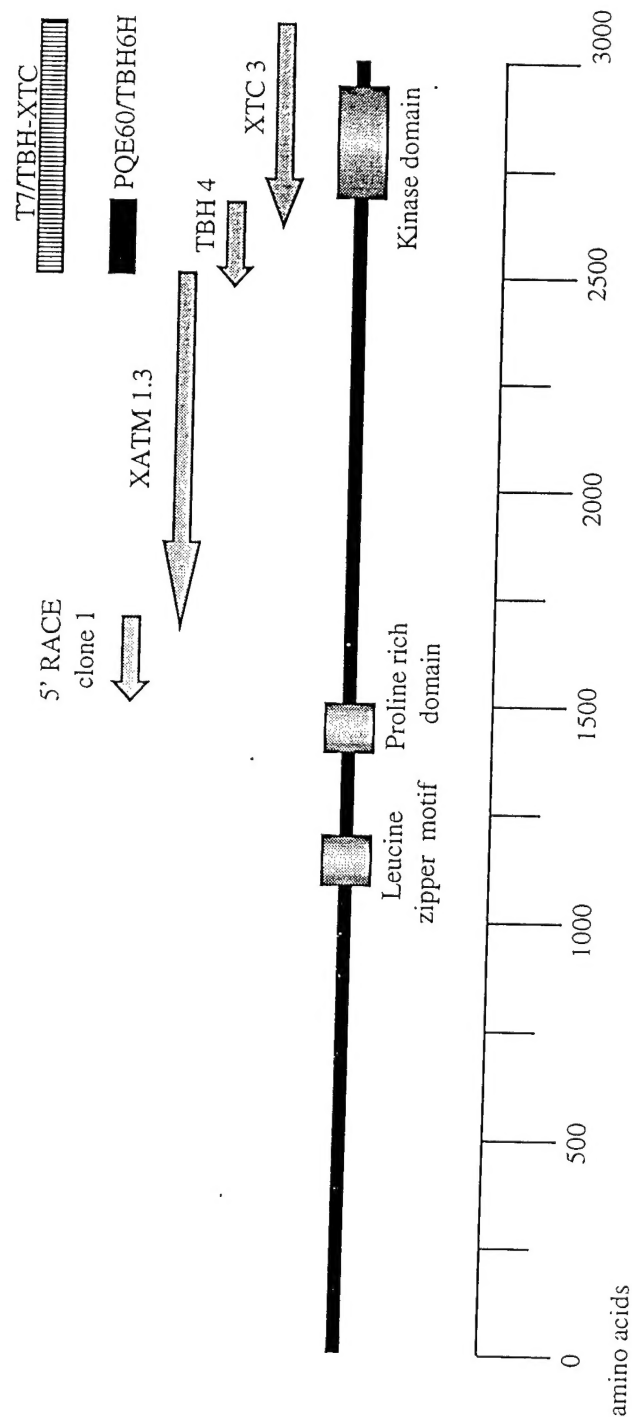
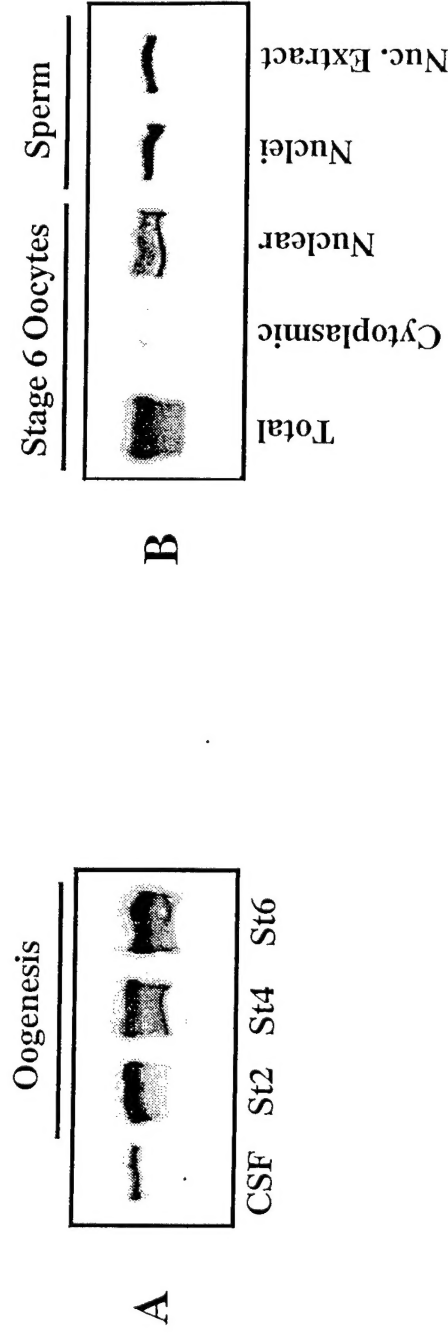


Figure 1B

X-ATM H-ATM Consensus	DVSTRSLLLC LDLLHRVCRA AVTHCEDALE CHHHVIVGSL DVSLSRSFSLC CDLLSCVCOT AVTYCKDALE NHLHVIVGTL DVS.RS..LC .DLL..CC.. AVT.C.DALE .H.HVIVG.L	40 1520	X-ATM H-ATM Consensus	MFQIKQYNPS QLGVSSEWLE EAQIFWDKEE PSLAREILKQ IFQIKQYNVS SCGVSEWLE EAQVFWAKKE QSLALSILKQ .FQIKQYN.. .GVSEWLE EAQ.FW.K.E .SLA..ILKQ	840 2310
X-ATM H-ATM Consensus	IPLANSQESI QEKVCOLLNF LVIENKNEN LYHTIKLLDP IPLVVEQVEV QKQVLDLLKY LVIDNKNEN LYITIKLLDP IPL...Q... Q..V.DLL.. LVI.NKNEN LY.TIKLLDP	80 1560	X-ATM H-ATM Consensus	MINKLEIKSF EVENDSRRLR LYAESLRRCG KWLAEETCLES MIKKLDASC -AANNPSLKL TYTECLRVCG NWLAETCLEN MI.KL..... .N...L.L .Y.E.LR.CG .WLAETCLE.	880 2348
X-ATM H-ATM Consensus	FPDIPLFKNL ROAHQRIKYS KGPFSLLKEI QNFLSVSVCD FPDHVVFCDL RITQOKIKYS RGPFSLLLEI NHFLSVSVYD FPD...F.K.L R...QRIKYS .GPFSLLEI .FLSVSV.D	120 1600	X-ATM H-ATM Consensus	PTVIMONYLE KAVEFAG-YS DGAGEKLQEG RMKAFSLSLAR PAVIMQTYLE KAVEVAGNYD GESSDELNRG KMKAFSLSLAR P.VIMQ.YLE KAVE.AG.Y.L..G .MKAFSLSLAR	919 2388
X-ATM H-ATM Consensus	SLPLTRLEGL NDLRKQLEQH KDQIKELVRD CQGTPOQSVI ALPLTRLEGL KDLRRQLELH KDQMDVIMRA SQDNPQDGM .LPLTRLEGL .DLR.QLE.H KDQ.....R. .Q..PD...	160 1640	X-ATM H-ATM Consensus	FSDAQYQRID NYMKSEFEN KQALLRKAKE EVGLIKQHKV FSDTQYQRIE NYMKSEFEN KQALLRKAKE EVGLLRHKI FSD.QYQRI. NYMKSEFEN KQALL..AKE EVGL...HK.	959 2428
X-ATM H-ATM Consensus	ASLVNLLQL SKNAVHQSNG KEVLEAVGSC LGELGPIDFS VKLVNLLQL SKMANHTGE KEVLEAVGSC LGVVGPIDFS ..LVVNLLQL SK.A..... KEVLEAVGSC LGE.GPIDFS	200 1680	X-ATM H-ATM Consensus	QNNRYTVKVE RELQLDECAI LALREDRKR LCKAVENYIS QTNRYTVKQV RELELDELAL RALKEDRKR LCKAVENYI Q.NRYTVKV. REL.LDE.A. .AL.EDRKR LCKAVENYI.	999 2468
X-ATM H-ATM Consensus	NIALQHKKD SVYLKADKVF EEKELQCVLV MLTLINNALT TIAIQSKDA S-YTKALKLF EDKELQWTFI MLTYLNNTLV .IA.Q...K. S.Y.KA.K.F E.KELQ.... MLT..NN.L.	240 1719	X-ATM H-ATM Consensus	CLVSGEEHDM WIFRLCSLWL ENSAVSDVNS MMRQDAQKIP CLLSGEEHDM WIFRLCSLWL ENSGVSEVNG MMKRDGMKIP CL.SGEEHDM W.FRLCSLWL ENS.VS.VN. MM..D..KIP	1039 2508
X-ATM H-ATM Consensus	DHCIOVRSAA ATCLKNILAT KTGAMFWEAC KDKGPEMLLY EDCVKVRSA VTKLKNILAT KTGHSWEIY KMTTDPMLY ..C..VRSAA .TCLKNILAT KTG..FWE.. K....PML.Y	280 1759	X-ATM H-ATM Consensus	SHKFLPLMYQ LAARMGTCKM GNGPFHDVLN NLIGRISMDH TYKFLPLMYQ LAARMGTCKM GGLGFHVLN NLISRISMDH ..KFLPLMYQ LAARMGTCKM G..GFH.VLN NLI.RISMDH	1079 2548
X-ATM H-ATM Consensus	LQPFRAKKK FLEVEEIQRE GALESLLDDP IWIPQENHE LQPFRTSKK FLEVPFDEK NPEGLDDIN LWIPLSENHD LQPPF...KK FLEV....E ..E.LDD.. .WIP..ENH.	320 1799	X-ATM H-ATM Consensus	PHHTLFILA LANANKDDQL MKAEAVKRSL LTKNAPKQIS PHHTLFILA LANANRDEFL TKPEVARRS ITKNVPKQSS PHHTLFILA LANAN.D..L .K.E...RSR .TKN.PKQ.S	1119 2588
X-ATM H-ATM Consensus	SWIKHLCTL LESGGVKSEV LLLLKPMCEV KADFCQAVVP IWIKTLTCAF LQSGGKCEI LQLLKPMCEV KDFCQTVLP .WIK.LTC.. L.SGG.K.E. L.LLKPMCEV K.DFCQ.V.P	360 1839	X-ATM H-ATM Consensus	QLDKERMEA RHIVDTIKKR RTDMVRDVER LCDAYITLAN QLDEDRTEAA NRICTIRSR RQPMVRSVEA LCDAYILAN QLD..R.EAA ..I..TI..R R..MVR.VE. LCDAYI.LAN	1159 2628
X-ATM H-ATM Consensus	YIVHNILLND SNQWRTLLS KNVORFTSC CRSLPSSRS YLHDIILLQD TNSWRNLLS THVGQFTSC LRHFSQTSRS Y..H.ILL.D .N..WR.LLS .LVQ.FFTSC .R.....SRS	400 1879	X-ATM H-ATM Consensus	MDANQWSQR NAIPIPSDQ ITKLNKLHDV VIPTMEIKVD LDATQWKTQ KGINIPADQ ITKLNKLEDV VVPTMEIKVD .DA.QWK.QR ..I.IP.DQ ITKL..L.DV V.PTHEIKVD	1199 2668
X-ATM H-ATM Consensus	ATPASSDSES EGVARGAVDI ASRRTMLTMV EHLRKQRRPV TTPANLDES EHFRCCLDK SQRTMLAVV DYMRQKRPS .TPA..DSES E...R...D. .S.RTML..V ...R.Q.RP.	440 1919	X-ATM H-ATM Consensus	PSGEYENLVT IVSFKPEFRL AGGLNLPKII DCVSGDGKER HTGEYGNLVT IQSFKAERFL AGGVNLPKII DCVSGDGKER ..GEY.NLVT I.SFK.EFRL AGG.NLPKII DCVSGDGKER	1239 2708
X-ATM H-ATM Consensus	SGTAFDDNFW LDNLYLEVAM AVQSCAAHFT ALLYSEIYTD SGTIFDNFV LDNLYLEVAK VAQSCAAHFT ALLYAEIYAD SGT.F.D.FW LDNLYLEVA. .QSCAAHFT ALLY.EIY.D	480 1959	X-ATM H-ATM Consensus	ROLVKGQDDL RQDAVMQVQV QMCNTLLQRN SETRKRKLTI ROLVKGRRDL RQDAVMQVQV QMCNTLLQRN TETRKRKLTI ROLVKG.DDL RQDAVMQVQV QMCNTLLQRN .ETRKRKLTI	1279 2748
X-ATM H-ATM Consensus	KVKQDGEORT SANRSNARKC LKFEESQTL DITGLSEKSK KKSMDQEE-- -----KRS LAPEESQST TISSLSEKSK K..D..... L.PREGSO.. .I..LSEKSK	520 1990	X-ATM H-ATM Consensus	RRYKVVPISH RSGVLEWCTG TVPIGEYLVN DKDGAHKRYR CTYKVVPISQ RSGVLEWCTG TVPIGEYLVN NEGGAHKRYR ..YKVVPIS. RSGVLEWCTG TVPIGE.LVN ..DGAHKRYR	1319 2788
X-ATM H-ATM Consensus	EETGISLQDL LMDIYRSIGE PDSLYCGGG KMLHPLARIR EETGISLQDL LLEIYRSIGE PDSLYCGGG KMLQPIRLR EETGISLQDL L..IYRSIGE PDSLYCGGG KML.P..R.R	560 2030	X-ATM H-ATM Consensus	PGDYGSLQCC RKMEVQQRG FEEKYQMFVN VCENFRPVFR PNDFSAPCC RKMEVQKKS FEEKYEVFMD VQCNFQPVFR P.D...CCQ .KMEVQ... FEEKY..F.. VC.NF.PVFR	1359 2828
X-ATM H-ATM Consensus	TYEHEAKWKG ALVTFDLEMN LPPVTRQAGI MQALQNFGLC TYEHEAMWKG ALVTYDLETA IPSSTRQAGI IQALQNLGLC TYEHEA.WKG ALVT.DLE.. .P..TRQAGI .QALQN.GLC	600 2070	X-ATM H-ATM Consensus	YFCHEKFLDP AMWFEKRLAY TRSVATSSIV GYIVGLGRH YFCHEKFLDP AIWFEKRLAY TRSVATSSIV GYILGLGRH YFCHEKFLDP A.WFEKRLAY TRSVATSSIV GYI.GLGRH	1399 2868
X-ATM H-ATM Consensus	HMLSTYLRGL EHENAESWE LQEIHFQAAW RNMQWDDSLP HILSVYKGL DYENKWCPE LEELHYQAAW RNMQWDHCTS H.LS.YL.GL ..EN..W..E L.E.H.QAAW RNMQWD....	640 2110	X-ATM H-ATM Consensus	VQNILIDEES AELVHIDLGV AFEQKILPT PETVFFRLTR VQNILINEQS AELVHIDLGV AFEQKILPT PETVFFRLTR VQNILI.E.S AELVHIDLGV AFEQKILPT PETVFFRLTR	1439 2908
X-ATM H-ATM Consensus	TKNETSGPGY HESLYRAVQS LRDKEFCGFH DHIKYARVKE VSKEVEGTSY HESLYNALQS LRDREFSTFY ESLKYARVKE ...E..G..Y HESLY.A.QS LRD.EF..F. ...KYARVKE	680 2150	X-ATM H-ATM Consensus	DIVDGMGITG VEGVFRRCCE KTMVNRNQ EALLTIVEVL DIVDGMGITG VEGVFRRCCE KTMVNRNSQ ETLTIVEVL DIVDGMGITG VEGVFRRCCE KTMVNRN.Q E.LLTIVEVL	1479 2948
X-ATM H-ATM Consensus	VEELCSGSL SVSYSLPALC RLQAIAGLAS VQGMFSRSIT VEEMCKRSLE SVSYSLPTLS RLQAIAGES IGELFSRSVT VEE.C..SLE SVSYSLP.L. RLQAIAGLAS .I.G.FSRS.T	720 2190	X-ATM H-ATM Consensus	LYDPLFDWTH NPLKALYLQO ---DEVDLNA TLGGDDPECN LYDPLFDWTH NPLKALYLQO RPEDETLHP TLNADQCECK LYDPLFDWTH NPLKALYLQO ...DE..L.. TL..DD.EC.	1516 2988
X-ATM H-ATM Consensus	DDGLKDTFLK WQSQSLQK SDFFLEFVL SLRSVILETL HRQLSEVYIK WQKSLQK SDFFFOEPI ALRTVILEIL ...L...K WQ..SQLK SDP.F.EP.. .LR.VILE.L	760 2230	X-ATM H-ATM Consensus	RNSCD-SQSV NKVAERVLRL LOEKLTVGEE GMVLVSGGVQ RNLSDIDQS DKVAERVLRL LOEKLGVEE GTVLVSGGVQ RN..D..QS. .KVAERVL.R LOEKL.VEE G.VLVSGGVQ	1555 3028
X-ATM H-ATM Consensus	LQEEKPOPSQ ESLKDILTK LLDLSRIARS AGHTOLPEKA MEKEMDNSQR ECIKDILTK LVELSILART FHTOLPERA ...E..... E..KDILTK L..LS..AR. .HTOLPE.A	800 2270	X-ATM H-ATM Consensus	NHLLQOAMPD KNLRLFPGW KAWVZ 1580 NLLIQOAIDP KNLRLFPGW KAWV 3052 N.LIQOA.DP KNLRLFPGW KAWV.	

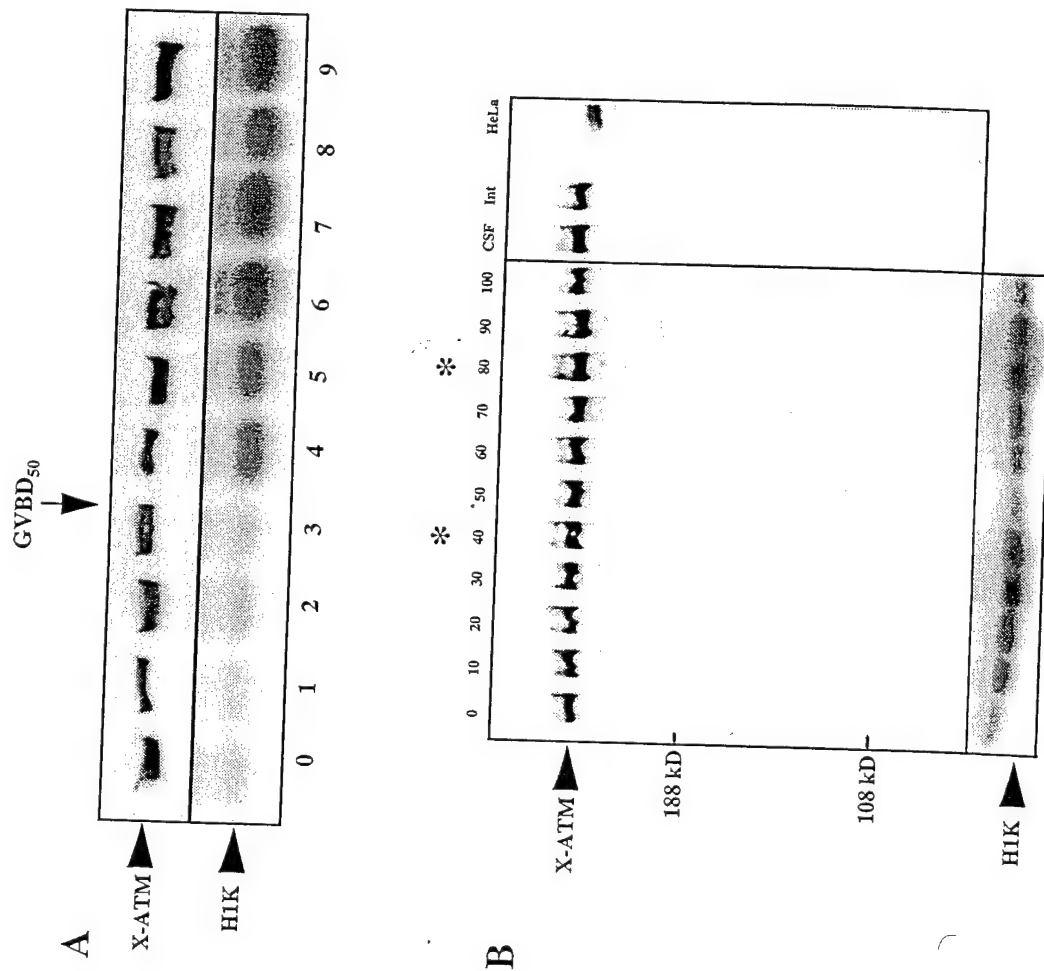
Sequence comparison of X-ATM and human ATM. Identical amino acids are indicated in bold letters below the comparison. The regions of the human sequence used to derive degenerate oligonucleotides are underlined (thin line). The PI3 kinase domain consensus region is underlined (thick line). The nucleotide sequence used to translate X-ATM has been deposited in the GenBank, the accession number is: AF174488



A: X-ATM during oogenesis. Manually dissected oocytes at different stages of vitellogenesis were collected. Equal amounts of protein were separated by electrophoresis and subjected to Western blotting. The stages of oogenesis are indicated below the lanes. The mobility was compared with that of X-ATM in a CSF extract (CSF). **B: Subcellular localization of ATM in the germline.** Protein samples from either complete stage VI oocytes (Total), stage VI oocyte cytoplasm (cytoplasm) or stage VI oocyte nuclei (nuclear) were processed for Western blotting. The two right lanes show a similar Western blot using either demembrated sperm nuclei or protein extracts prepared from nuclei.

Figure 2

Figure 3



A: X-ATM expression throughout the meiotic cell cycle. Western blot analysis of oocytes progressing synchronously through meiosis was performed. $t=0$ is the time of addition of progesterone. GVBD₅₀ indicates the G2/M transition, the * indicates the interphase between metaphase of meiosis I and metaphase of meiosis II. X-ATM Western blot: top panel, Histone H1 kinase activity of the same samples : bottom panel. **B: X-ATM during the mitotic cell cycles** ATM levels during S phase and mitosis were compared by western blot analysis of "cycling extracts" prepared from *Xenopus* eggs. X-ATM levels were monitored by 6% SDS-PAGE and western blot analysis of extracts over a period of 100 minutes (top panel), corresponding to two cell cycles as determined by analysis of cdc2/cyclin B kinase activity (bottom panel). M phase, i.e. the kinase activity peaks, is indicated by *. Comparison of a mitotic extract (CSF), and an interphase extract (INT), also indicated a similar amount of ATM in these different cell cycle phases. For comparison, a HeLa whole-cell lysate was also analyzed on this gel. Human ATM antibody (Gately, D.P) recognizes a band with a slightly faster mobility than *Xenopus* ATM.

Figure 4

X-ATM protein following DNA damage. The level of ATM in extracts from control and γ -irradiated embryos between stage 8 and 10.5 was compared. Embryos were γ -irradiated (40 Gy) using a ^{60}Co source at stage 2. Embryos were collected and crushed at the indicated stages, and the soluble extract was analyzed by 6% SDS-PAGE followed by Western blot analysis. ATM in a mitotic extract (CSF) is also shown.

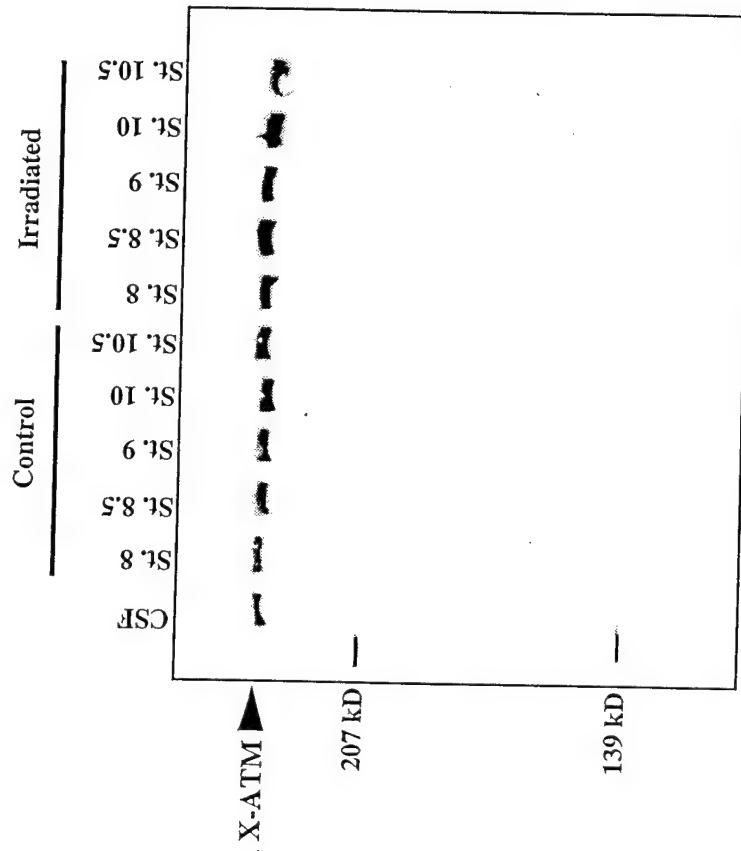
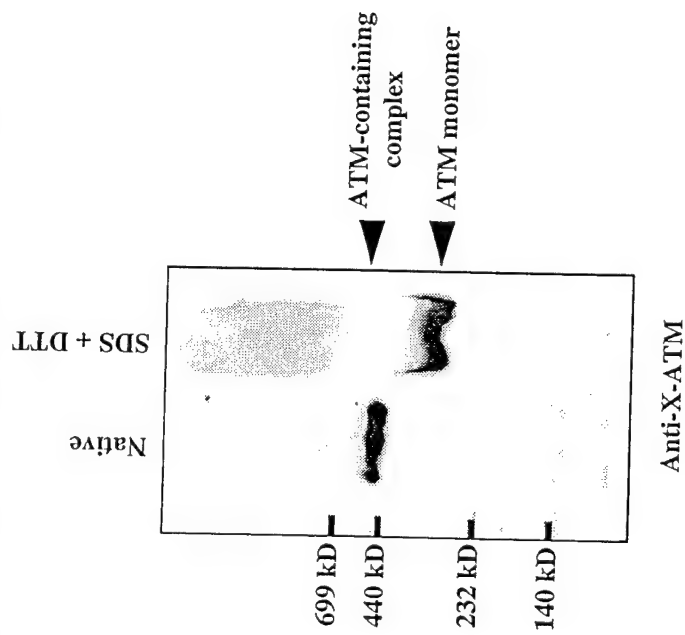


Figure 5

X-ATM complexes 200 μ g of egg extract were electrophoresed under native conditions and immunoblotted with X-ATM antibody (first lane). A band of approximately 450-500kD corresponding to the complex containing X-ATM is observed as indicated. The same sample was electrophoresed in the same polyacrylamide gradient gel under reducing and denaturing conditions (second lane). The immunoreactive band migrates at 370kDa, the molecular weight of monomeric ATM.



**Isolation and Characterization of Xenopus ATM (X-ATM):
Expression, Localization, and Complex Formation During Oogenesis
and Early Development.**

Kirsten Robertson, Carmel Hensey and Jean Gautier*.

Department of Genetics and Development and Department of Dermatology, College of Physicians
and Surgeons of Columbia University, 630 West 168th Street, New York, NY 10032.

*Corresponding author.

Telephone: (212) 305 9586

Fax: (212) 305 7391

E-mail: jg130@columbia.edu

ABSTRACT

ATM, the gene product mutated in Ataxia Telangiectasia (A-T) encodes a 350-kDa protein involved in the regulation of several cellular responses to DNA breaks. We used a degenerate PCR-based strategy to isolate a partial clone of X-ATM, the *Xenopus* homologue of human ATM. Sequence analysis confirmed that the clone was most closely related to human ATM. *Xenopus* ATM protein (X-ATM) is 85% identical to human ATM within the kinase domain and 71% identical over the carboxyl-terminal half of the protein. Polyclonal antibodies raised against recombinant X-ATM are highly specific for the ATM protein and recognize a single polypeptide of 370-kDa in oocytes, embryos, egg extracts and a *Xenopus* cell line. We found that X-ATM was expressed maternally in eggs and as early as stage II pre-vitellogenic oocytes, and the protein and mRNA were present at relatively constant levels throughout development. Subcellular fractionation showed that the protein was nuclear in both the female and male germlines. The level of X-ATM protein did not change throughout the meiotic divisions or the synchronous mitotic cycles of cleavage stage embryos. In addition, we did not observe any change in the level or mobility of X-ATM protein following γ -irradiation of embryos. Finally, we also demonstrated that X-ATM was present in a high molecular weight complex of approximately 500 kDa containing the X-ATM protein and other, as yet unidentified component(s).

INTRODUCTION

Ataxia Telangiectasia (A-T) is an autosomal recessive disease which displays a complex phenotype (Boder & Sedgwick, 1970; Shiloh, 1998). Patients exhibit a progressive cerebellar ataxia, in addition to severe immune deficiencies, gonadal atrophy, telangiectases, increased risk for cancer, particularly lymphomas, and radiation sensitivity. Additionally, carriers are suspected to be prone to other cancers including breast cancer (Athma *et al.*, 1996; Chen *et al.*, 1998; Stankovic *et al.*, 1999; Swift *et al.*, 1990; Yuille & Coignet, 1998).

Cells from A-T patients show increased radiosensitivity to ionizing radiation (Lavin & Shiloh, 1997), increased chromosomal loss and breakage, and abnormal telomere morphology (Smilenov *et al.*, 1997; Vaziri *et al.*, 1997). Furthermore, these cells are defective in cell cycle checkpoints in G1, S and G2 phases of the cell cycle (Beamish *et al.*, 1996; Hoekstra, 1997; Meyn, 1995). Although complex, the cellular phenotype of A-T points to a defect in handling DNA breaks formed either following damage or subsequent to normal physiological processes such as meiotic recombination and the maturation of the immune system.

The identification of a single mutated gene called ATM (Ataxia Telangiectasia Mutated) as the molecular basis for the phenotype has allowed a better understanding of both ATM function and the A-T pleiotropic phenotypes (Savitsky *et al.*, 1995; Taylor, 1998). ATM is a nuclear phosphoprotein (Chen & Lee, 1996; Scott *et al.*, 1998) and is a Serine/Threonine protein kinase for which the c-abl proto-oncogene, the replication protein RPA and the p53 tumor-suppressor gene have been identified as substrates in vitro (Banin *et al.*, 1998; Baskaran *et al.*, 1997; Canman *et al.*, 1998; Gately *et al.*, 1998; Khanna *et al.*, 1998; Nakagawa *et al.*, 1999; Shafman *et al.*, 1997). The identification of these potential substrates have helped place ATM in a signal transduction pathway in which it could function in a cell cycle checkpoint through c-abl and p53.

Cells respond to DNA damage by activating checkpoint pathways that delay progression through the cell cycle (Hensey & Gautier, 1995). ATM functions in the checkpoint pathway activated by DNA damage. It has been shown that p53 is phosphorylated in an ATM-dependent fashion following DNA damage suggesting that p53 could be a direct substrate for the ATM protein kinase (Banin *et al.*, 1998; Canman *et al.*, 1998). Activation of p53 following DNA

damage also involves dephosphorylation events that are dependent upon ATM activity (Waterman *et al.*, 1998). In addition, studies in yeast and in mammalian cells also suggest that ATM might function as an upstream regulator of the Chk1 and Chk2 kinases (Brown *et al.*, 1999; Chen *et al.*, 1999; Matsuoka *et al.*, 1998). However, the exact in vivo biochemical function of the ATM protein and its physiological substrates still remain elusive. In particular, the gap between our in vitro biochemical knowledge of the protein and its relationship with both cellular and patient phenotypes still awaits investigation.

A mouse model for ATM deficiency was created in several laboratories by specific germline inactivation of the ATM gene (Barlow *et al.*, 1996; Elson *et al.*, 1996; Herzog *et al.*, 1998; Xu *et al.*, 1996; Xu & Baltimore, 1996). Fibroblasts isolated from ATM^{-/-} mice display similar cellular phenotypes to those observed in cells from A-T patients. As is the case with A-T patients, ATM deficient mice display a variety of growth defects, meiotic defects, immunological abnormalities, acute radiation sensitivity and cancer predisposition, confirming the pleiotropic roles of ATM.

Xenopus laevis is a powerful model system for both biochemical studies of cell cycle and checkpoint regulation as well as for developmental studies. We decided to use *Xenopus* as a novel model system to study both the biochemical role of ATM and its function during development. The influence of DNA replication inhibition upon cell cycle progression (DNA replication or S-phase checkpoint) has been studied in detail in *Xenopus* egg extracts. First, it has been shown that threshold inhibition of DNA replication can elicit cell cycle arrest in egg extracts and prevent entry into mitosis (Dasso & Newport, 1990). Furthermore, both cdc25 and wee1 have been identified as modulators of this checkpoint. Upstream of cdc25, the protein kinase Chk1 and the 14-3-3 adapter protein have also been shown to be essential for cell cycle arrest (Kumagai *et al.*, 1998a; Kumagai *et al.*, 1998b).

To enable such studies we have obtained a partial clone of the *Xenopus* homologue of the ATM protein. We present its detailed expression patterns during the meiotic and the mitotic cell cycles as well as during development. We show that X-ATM is a maternal protein expressed as early as stage II oocytes and that ATM is exclusively nuclear in the germline. Finally, we present evidence that ATM exists in a large molecular weight complex in extracts derived from eggs.

RESULTS

Isolation of a partial clone of the *Xenopus* homologue of ATM.

A protein alignment of the putative kinase domain of human ATM, *S. pombe* rad3, human ATR, *S. cerevisiae* TEL1, and *D. melanogaster* mei41 was used to identify regions of homology within the kinase domain of ATM and TEL1, that were not present in ATR, rad3 or mei41. Four amino acid stretches were identified and used to design degenerate oligonucleotides (see methods and Figure 1B). A 480 bp cDNA fragment was isolated by RT-PCR (Figure 1A) and sequencing of the fragment confirmed that it was most closely related to human ATM (Figure 1B). Using library screening, ATM-specific library construction, and 5'RACE (Figure 1A) additional sequence corresponding to approximately 50% of the predicted *Xenopus* ATM ORF was obtained.

An alignment of the carboxyl-terminal half of X-ATM with human ATM is shown in figure 1B. ATM is highly conserved between *Xenopus* and human with an overall identity of 71% in the region we have sequenced. Within the kinase domain the identity is 85% while the region upstream of the kinase domain (amino acids 1 to 1100 of X-ATM) shows 66% identity to the human protein. In contrast, when this region from X-ATM (a.a. 1 to 1100) is aligned with *S.c.* TEL1, *S.p.* rad3, *D.m.* mei41 or *H.s.* ATR, only short stretches of weak homology (ranging from 20 to 24%) are detected.

Characterization of X-ATM antibodies

Using an *E.coli* expression vector, we produced a 20-kDa fragment of the X-ATM cDNA corresponding to the region immediately 5' of the protein kinase domain (PQE60/TBH6H of Figure 1A). We chose this region as it had been previously shown to elicit a good immune response for human ATM (Brown *et al.*, 1997). Additionally, we wanted to avoid raising antibodies against the conserved kinase domain which could potentially cross-react with other members of the ATM/PI3 kinase family. This 20-kDa polypeptide was expressed in *E. coli* and was used as an antigen for antibody production in rabbits (See Materials and Methods).

Programmed reticulocyte lysate containing the 65-kDa carboxyl terminal portion of X-ATM (T7/TBH-XTC of Figure 1A) was used to screen the different antisera. Sera from both rabbits

showed strong cross-reactivity with the translated product (data not shown) and recognized a protein of approximately 370-kDa in *Xenopus* egg extracts (Figure 2A, lanes 1 and 2). Due to the presence of a 210-kDa cross-reacting polypeptide in serum A (Figure 2A, lane 1), we used serum B for the remaining experiments. Preincubation of the antiserum with the 20-kDa polypeptide used for immunization eliminated the signal, confirming the identity of the protein recognized by the antiserum (data not shown). Moreover, we were able to immunoaffinity purify the X-ATM antibody from rabbit B using the 20-kDa polypeptide coupled to agarose. This purified antibody, which was used in all further experiments, detected a single polypeptide in *Xenopus* egg extracts that migrated slightly slower than human ATM (compare lanes 3 and 4, figure 2A). It also immunoprecipitated a fraction of the X-ATM protein from extracts as shown in figure 2B (lane 2).

Expression of X-ATM during early development.

Little is known about ATM expression during the early phases of vertebrate development. We therefore examined the temporal expression of both X-ATM mRNA and protein between fertilization and the swimming tadpole stage in *Xenopus*. Figure 3A shows an RNase protection assay for the XTC cell line (Pudney *et al.*, 1973) and the indicated stages of development. X-ATM mRNA was detected in unfertilized eggs and expressed at relatively constant levels throughout cleavage, gastrulation, neurulation and the tadpole stage, although we consistently detected higher X-ATM mRNA levels in unfertilized eggs. Since transcription of zygotic genes does not start until the midblastula transition (St. 8-9), this demonstrates that X-ATM mRNA is maternally inherited. The temporal expression of X-ATM protein during similar developmental stages is shown in figure 3B. The protein is expressed maternally and throughout development where we observe a single cross-reacting polypeptide at all stages. Some variability in the X-ATM protein amounts was detected, particularly during neurulation. This might reflect developmentally regulated changes in the amount of X-ATM. Alternatively, this could also be due to the subcellular localization of the protein leading to different yields following embryonic extract preparation. We estimated the concentration of X-ATM to be 200 nM in egg extracts and 40 nM in unfertilized eggs (see Materials and Methods).

Subcellular localization of the ATM protein in the germline.

X-ATM is present in egg extracts and in unfertilized eggs (Figure 2A and 3B). We examined whether the protein was already present in the oocyte as opposed to being synthesized during meiotic maturation as has been described for some proteins (Sagata *et al.*, 1988). *Xenopus* oocytes were manually dissected from pieces of ovary and protein extracts were made at the stages of oogenesis indicated (Dumont, 1972). X-ATM is already present in previtellogenic oocytes (stage II) and persists throughout oogenesis (Figure 4A). Although the concentration of X-ATM per mass of tissue does not vary significantly throughout oogenesis, the mass per oocyte increases dramatically since the volume of the oocytes increases by a factor of 10^3 between stages I-II and stage VI.

Stage VI *Xenopus* oocytes contain a large (200 μm diameter) nucleus, known as the germinal vesicle (GV), which can be used to store nuclear proteins important for later development (Dreyer & Hausen, 1983). The GV can be isolated surgically from its surrounding cytoplasm, allowing the preparation of pure cytoplasmic and nuclear fractions from oocytes (Ford & Gurdon, 1977). We demonstrate that X-ATM is entirely nuclear in stage VI oocytes (Figure 4B). Moreover, using either demembranated sperm nuclei, or a nuclear extract prepared from these nuclei following their incubation in extracts (Walter *et al.*, 1998), we show that X-ATM is also nuclear in the male germline.

Expression of X-ATM protein throughout the meiotic and mitotic cell cycles.

In *Xenopus* oocytes the highly synchronous meiotic cell cycle is easily studied *in vitro*, where progesterone induced oocyte maturation culminates in the completion of meiosis I and the progression to metaphase of meiosis II. To determine whether X-ATM protein levels fluctuated during the meiotic cell cycle, we performed Western blots on protein samples from oocytes synchronously undergoing meiosis. X-ATM protein levels remained constant throughout meiosis (Figure 5A). In this experiment, prophase-arrested oocytes were induced to undergo meiotic maturation *in vitro* by progesterone. Nuclear envelope breakdown took place at 3.5 hrs, meiosis I occurred at 6 hrs and the oocytes were arrested in metaphase of meiosis II by 9 hrs.

Next, we followed X-ATM levels throughout the mitotic cell cycle which oscillations were assessed by following *cdc2/cyclinB* activity. The naturally synchronous cell divisions of the early embryo allow the preparation of cell free extracts that undergo cell cycle oscillations in vitro. Using such extracts we examined X-ATM protein levels throughout these highly synchronized cell cycles. ATM is expressed throughout the cell cycle and its levels remain constant over the course of two cell cycles (Figure 5B). Additionally, there was no change in the electrophoretic mobility of X-ATM.

X-ATM protein in γ -irradiated eggs

In *Xenopus*, irradiation of cleavage stage embryos leads to massive and synchronous apoptosis at the onset of gastrulation (St. 10.5) (Anderson *et al.*, 1997; Hensey & Gautier, 1997). To assess whether this response correlated with changes in the amounts or the electrophoretic mobility of X-ATM, we compare the levels of X-ATM in protein samples from untreated and γ -irradiated embryos between stage 8 and 10.5. X-ATM protein levels were similar in control and irradiated embryos. In addition no change in the mobility of the protein was detected (Figure 6).

X-ATM is present in a protein complex in *Xenopus* extracts.

The carboxyl-terminal kinase domain of ATM represents a small portion of this large protein. Little is known about the function of the domain(s) outside the kinase region but support for its importance comes from both genetic analysis of ATM mutants (Concannon & Gatti, 1997) as well as the finding that a putative leucine zipper region of ATM behaved as a transdominant negative (Morgan *et al.*, 1997). A central proline rich domain has already been shown to interact with the *c-abl* SH3 domain (Shafman *et al.*, 1997), and the remainder of the protein is suspected of playing regulatory functions by interacting with other proteins. To determine whether X-ATM might be associated with other proteins in *Xenopus* extracts we analyzed extracts by native gel electrophoresis. We detected a native, high molecular weight X-ATM-containing complex with an estimated size of 500-kDa (Figure 7). In addition, using sucrose gradient centrifugation, we detected a high molecular weight form of ATM in extracts consistent with our electrophoresis analysis (data not shown).

DISCUSSION

Xenopus ATM

We report the cloning of a *Xenopus* homologue of ATM, the gene mutated in Ataxia-Telangiectasia. Although we have not yet isolated the full-length clone, sequence comparison spanning 50% of the predicted ORF, establishes clearly that we have isolated a *Xenopus* ATM homologue rather than a member of the PI3-kinase family. The amino acid levels of identity between X-ATM and human or mouse ATM are 71% and 69%, respectively while human ATM is 85% identical to mouse ATM over the same half of the protein. In comparison, there is only weak homology outside the kinase domain between X-ATM and ATR, mei41 or rad3, three genes which are forming the "ATR/rad3" branch of this family of protein kinases (Hoekstra, 1997).

The high degree of conservation between X-ATM and ATM within the kinase domains (85% identity) strongly suggests that the catalytic activity of these two proteins will be conserved. The decreasing levels of identity outside the catalytic domains could reflect potential differences in regulation of these proteins between species.

Mutations in the ATM gene are not clustered in a specific area of the molecule or restricted to a limited numbers of residues, instead they span the entire ORF of the molecule. This has made it difficult to discriminate between true mutations and polymorphisms. The availability of X-ATM sequence might help resolve some of these issues by identifying mutations in conserved residues that have been originally classified as polymorphisms. Conversely, it might help confirm the location of polymorphisms in non-conserved residues.

Cell cycle patterns of X-ATM expression

We present a detailed analysis of the expression of X-ATM throughout both meiotic and mitotic cell cycles. These cell cycles are naturally synchronous in *Xenopus* and allowed the cell cycle expression of ATM to be followed in undisturbed cell cycles. We find no evidence for cell cycle oscillation in the level or the electrophoretic mobility of ATM protein during the mitotic cell cycles in cell-free extracts or during the meiotic divisions of the oocyte. This confirms previous results for human ATM obtained in cells that have been synchronized in culture (Brown *et al.*,

1997; Gately *et al.*, 1998). Due to the large size of X-ATM, small variations in mobility might not be detectable despite the use of very resolutive PAGE. In addition, ATM could be modified in ways that does not affect its electrophoretic mobility. Therefore we cannot rule out that X-ATM protein could be modified in a cell cycle dependent manner.

Developmental expression of X-ATM

We found that X-ATM was expressed maternally, very early during oogenesis. This might reflect the need for ATM function in growing oocytes and during meiosis. Later during embryonic development we did not observe dramatic changes in the levels of mRNA or protein which is consistent with X-ATM playing a role in all cell types throughout development. RNase protection assays show the presence of a second protected fragment at stages 9 and 10 of smaller size than the expected protected fragment. This could be due to the 2 different mRNA species generated by alternative splicing. Alternatively, a stable secondary structure could exist at either end of the protected fragment giving rise to the observed pattern. Since alternative mRNA splicing has not been observed within the ORF of human ATM (Savitsky *et al.*, 1995) we favor the second hypothesis.

We also followed the protein expression patterns of ATM in embryos that have been irradiated by ionizing radiation. We previously reported that such an insult induces a synchronous apoptotic program in *Xenopus* embryos, manifest at the onset of gastrulation (Anderson *et al.*, 1997; Hensey & Gautier, 1997). In such embryos destined to die by apoptosis we did not observe any changes in the levels of X-ATM. Moreover, when we followed X-ATM in *Xenopus* apoptotic extracts containing active caspase (as seen by PARP cleavage), we did not observe X-ATM cleavage despite the presence of a good consensus site for caspase cleavage (towards the C-terminal-end) of the X-ATM molecule (DIVD) (data not shown).

Localization

We clearly demonstrated that X-ATM is exclusively nuclear in the germline. *Xenopus* oocyte nuclei can be manually isolated from their surrounding cytoplasm with virtually no cross contamination. This provides unequivocal evidence for the subcellular localization of X-ATM in this system. Although X-ATM isolated from oocytes migrated with the same mobility on PAGE as

X-ATM isolated from eggs (see figure), we occasionally observed a second slower migrating band on Western blots from oocytes early during vitellogenesis (data not shown).

As meiotic maturation takes place and the nuclear envelope breaks down, X-ATM becomes cytoplasmic. In the unfertilized egg, corresponding to the second meiotic metaphase, the protein is cytoplasmic as seen by its presence in cell-free extracts. The nuclear localization of the protein in oocytes might reflect the fact that X-ATM could be essential for the proper completion of meiosis as is the case in mammals (Barlow *et al.*, 1998; Xu *et al.*, 1996). However, during the early cleavages of the embryos, the rapid divisions lack cell cycle checkpoints and are relatively insensitive to either DNA replication inhibitors or DNA damage (Hensey & Gautier, 1997). It will be interesting to determine whether exclusion of ATM from the nucleus correlates with the lack of cell cycle checkpoints and at what time during development X-ATM becomes nuclear again.

It is interesting to compare the expression and localization of X-ATM with that of *Xenopus* p53 since in mammalian systems p53 has been shown to be a target and a substrate for the ATM protein kinase. *Xenopus* p53 is structurally and functionally related to human p53 (Cox *et al.*, 1994; Soussi *et al.*, 1987; Wang *et al.*, 1995). Like X-ATM, p53 is present in oocytes and synthesized during early oogenesis (Tchang *et al.*, 1993). However in contrast to what we observe for X-ATM, *Xenopus* p53 is entirely cytoplasmic in the oocyte. The difference in sub-cellular localization between p53 and X-ATM might provide a partial explanation for the unusually high levels of p53 protein observed in *Xenopus* oocytes and embryos.

Complex formation

We also clearly demonstrate that ATM is present in at least one larger complex than the monomeric form. The non-denaturing gels we used in these experiments allowed us to clearly resolve a complex of approximately 500 kDa, which could be disrupted following SDS treatment. It is possible that other larger complexes also exist but cannot be resolved using PAGE. We confirmed these findings using sucrose gradient centrifugation of egg extracts followed by Western blotting. We found that X-ATM was found in a high molecular weight complex sedimenting around 600 kDa (data not shown).

The finding that X-ATM is associated with other partner(s) in the simple, unregulated cell cycles of *Xenopus* cell-free extracts suggest that X-ATM might always require the association with regulatory subunits to perform its functions. ATM has been shown to interact with c-abl which is also a substrate for the kinase. It was also demonstrated that ATM interacts with β -adaptin, a protein involved in clathrin-mediated endocytosis of receptors (Lim *et al.*, 1998). The identity of the *Xenopus* ATM partner(s) awaits further investigation.

MATERIALS AND METHODS

Cloning of *Xenopus Atm*

Degenerate RT-PCR: Four Degenerate primers were designed from regions of amino acid identity between the kinase domain of ATM and its yeast homologue TEL 1, with a slight bias towards the human sequence. Primers 1 and 2 were in the 5'-3' direction. Primers 3 and 4 were in the 3'-5' direction. The primer sequences were as follows:

Primer 1: 5'-GCGCGGATCCGA(CT)GA(CT)(CT)T(AGCT)(AC)G(AGCT)CA(AG)GA(CT)-3' (amino acids DDLRQDA, positions 2719 to 2726 in ATM)

Primer 2: 5'-GCGCGGATCCT(AGCT)ATG(GC)A(AG)CA(AG)GT(AGCT)TT(CT)-3' (amino acids MQQVFQ, positions 2727 to 2733 in ATM)

Primer 3: 5'-GCGCATCGATA(CT)(AGCT)CC(AGCT)A(AG)(AG)TC(ATG)AT(AG)TG-3' (amino acids HIDLGV, positions 2886 to 2892 in ATM)

Primer 4: 5'-GCGCATCGATTT(AGCT)CC(CT)TG(AGCT)TC(AG)AA(AGCT)GC-3' (amino acids AFEQGK, positions 2892 to 2898 in ATM)

Total RNA was isolated from *Xenopus* Tissue Culture (XTC) cells (RNAzol B, Tel-Test) and was subjected to a reverse transcription reaction. The cDNA product was used as a template in PCR with primers 1 and 4 in a standard PCR reaction. A fragment of approximately 480 bp was amplified, and reamplified with nested degenerate primers 2 and 4.

Library Screens: A λ Zap library from XTC cells was screened using the 480 bp PCR fragment as a probe and a high stringency hybridization was performed. Following 3 successive rounds of screening the clone XTC 3 was isolated containing a 2.1 kb insert including 600 bp of 3' UTR.

A further 500 bp of *X-Atm* was cloned upon screening of a second λ Zap library from Stage 25 *Xenopus* embryos (A gift from Dr. Hemmati-Brivanlou and Dr. Harland). The TBH 4 clone was 1 kb in size and partially overlaps with the 5' 500 bp of clone XTC 3 (see Figure 1A)

X-Atm Library construction: A Zap Express library was constructed using the Zap Express cDNA Gigapack III Gold Cloning kit, (Stratagene). Messenger RNA was isolated from XTC cell total RNA, and subjected to first strand synthesis using a primer specific for *X-Atm* (instead of the oligo DT primer provided) containing a XhoI site 5'-GAGAGAGAGACTA GTCTCGAGTGTGTCGACAATGTGACGTGC-3'. All cDNA's with molecular weights above 1 kb were excised, packaged into λ arms and the library titrated.

Subsequent screening of 20,000 plaques revealed a 2.5-kb clone, *X-Atm1.3* that overlaps with the 5' 210 bp of the TBH 4 clone (Figure 1A).

5' RACE: mRNAs were isolated from XTC cells as previously mentioned and reverse transcribed. Second strand synthesis and adapter ligation was carried out using the Marathon 5' RACE kit (Clontech). Long distance PCR was performed on the cDNA. After 3 rounds of PCR with nested primers an 800 bp product, clone RACE 1, was generated and resulted in a further 500 bp of new sequence (350 bp of this clone overlaps with the 5' of clone *X-Atm1.3*, see Figure 1A).

The assembly of all preceding clones provided 5.3 Kb of sequence corresponding to the 3'UTR and 50% of the predicted ORF of the cDNA. Amino acid sequence alignments and alignment scores were performed using Geneworks software.

RNase Protection Assay

The RNase protection assay was performed according to the protocol described in the RPAII kit (Ambion). A 480-bp fragment from clone TBH 4 (nucleotide position 5 to 485) was used as a probe (Figure 1A). The probe was hybridized with 15 μ g of XTC cell total RNA and with RNA from samples of 10 *Xenopus* eggs or embryos, stages 5, 9, 10, 17 and 26. After hybridization the samples were digested with RNase A and H, and analyzed by PAGE.

Bacterial Expression of X-ATM Fragment and Generation of X-ATM Antibodies

A 500 bp fragment from *X-Atm* clone TBH 4 (positions 5 to 485) was amplified by the forward primer 5'-GACGTCCTTAATAATCTGATT-3' upstream of an internal Nco I site and the reverse primer 5'-GCGCGGATCCGAGATTAAGGCCCCCGGCCAG-3' which contains a Bam HI site (Figure 1A) and cloned into the Nco I and Bam HI sites of the His₆ Expression vector PQE60.

We also cloned the 3' 1.5kb of the *X-Atm* ORF into a T7 vector that allowed the production of a 65-kDa polypeptide in rabbit reticulocyte lysate (T7/TBH-XTC).

X-ATM was expressed in *E. coli* and affinity purification of the denatured peptide was carried out as described in the QIAexpress protocol (Qiagen). 2 mg of protein was run on a 15% preparative polyacrylamide gel, the band excised from the gel and used to immunize rabbits.

Sera were tested by western blotting against the in vitro translated X-ATM. Sera from two rabbits gave a strong signal and the antibodies from rabbit B were further purified on an affinity column prepared by coupling the 20 kDa X-ATM protein fragment to CNBr sepharose beads.

Protein Sample Preparation

Cycling and CSF extract: Cycling extracts were prepared from unfertilized *Xenopus* eggs, as described in (Murray, 1991).

Embryos: Batches of 20 embryos from the indicated stages were collected and snap frozen in liquid nitrogen. The embryos were crushed in a modified RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 0.5% Nonidet P-40, 0.05% SDS) containing pepstatin, leupeptin, aprotinin and AEBSF (10µg/ml each). 100 µg of protein from each sample used in western blots.

Oocytes: Stages II, IV, and VI (Dumont, 1972) were gently crushed in 150 mM NaCl, 50 mM Tris, pH 7.4 and processed in the same way as embryos. Oocyte maturation, i.e. meiosis, was induced in vitro by 10 µM progesterone in MBS-H (Gautier & Maller, 1991).

Cells: XTC cells were washed in ice-cold PBS, pelleted and resuspended in RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Nuclear Protein Extracts (NPE) were prepared according to (Walter *et al.*, 1998).

Western Blotting

Protein samples were separated under denaturing conditions on 20 cm, high resolution 6% SDS polyacrylamide gels, and transferred to nitrocellulose. After blocking in 5% milk in PBS, membranes were incubated with either affinity purified X-ATM antibody or human ATM antibody AT 1.8 (a gift from Dr. T. Yen), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized using enhanced chemiluminescence (Amersham).

Quantitation of X-ATM in *Xenopus* embryos

Linear T7/TBH-XTC clone (Figure 1A) was used as a template in an *in vitro* transcription/translation reaction with 2 μ l of 35 S methionine of known specific activity. 1 μ l samples from the completed reaction were counted in a scintillation counter to determine the average number of counts corresponding to the calculated number of moles of 35 S-Met. The amount of 35 S-Met incorporated into the X-ATM band was then determined by electrophoresing 1-3 μ l of the translation reaction on a 10 % SDS-PAGE gel and transferring to nitrocellulose. The radiolabelled band was excised and counted, and counts were converted to pmoles of 35 S per μ l of the translation. Western blots of both the translation products and CSF extract were carried out in parallel and the band intensities of different dilutions were compared and hence, knowing the number of methionines in the translation product, we were able to determine the molarity of X-ATM in CSF extract. This was extrapolated to embryos by comparing band intensities in western blots containing CSF and extracts from a known number of embryos resulting in an estimation of the Molar concentration of X-ATM per embryo

Native gradient gel electrophoresis

10 μ l of interphase extract were either suspended in TBE containing 10 % glycerol or a modified buffer also containing 3 % SDS and 100 mM DTT. Samples in the SDS/DTT containing buffer were heated to 80°C for 5 minutes. Proteins were then separated on a non-denaturing linear gradient polyacrylamide gel in 1X TBE overnight. Before transferring proteins to nitrocellulose the gel was soaked in SDS buffer (48 mM Tris, 39 mM glycine, 0.25 % SDS) for 30 minutes at room

temperature with agitation, followed by a 30 minute soak at 80°C (Hendrickson *et al.*, 1996). Proteins were probed with the X-ATM antibody as previously described.

FIGURE LEGENDS

Figure 1.

A: ATM domains and cloning strategy.

The human ATM protein is represented by a thick black line, specific domains are indicated. X-ATM clones are indicated by arrows. T7/TBH-XTC denotes the in vitro translated fragment of X-ATM used to screen sera from rabbits A and B. PQE60/TBH6H was used in antigen production and part of this region was used in RNase protection assays (construct T7/TBH-XTC)

B: Sequence comparison of X-ATM and human ATM.

50% of the predicted sequence of *Xenopus* ATM (X-ATM) was aligned with the amino acid sequence of human ATM (H-ATM) using Geneworks software (Intellegentics). Identical amino acids between the 2 proteins are indicated in bold letters below the comparison. The regions of the human sequence that were used to derive degenerate oligonucleotides are underlined (thin lines). The PI3 kinase domain consensus region is underlined (thick line). The nucleotide sequence that was used to translate X-ATM has been deposited to GenBank, the accession number is: AF174488.

Figure 2. Characterization and specificity of antibodies against X-ATM.

A: Specificity of the crude serum and the affinity purified antibody for X-ATM.

100 µg of *Xenopus* egg extract was electrophoresed (6 % SDS-PAGE) and Western blotted with the crude serum A (lane 1), the crude serum B (lane 2) or the affinity purified antibody from serum B (lane 3). In all cases a polypeptide of 370kDa was detected. Lane 4 is a human cell extract (HeLa) probed with an anti-human ATM antibody (Gately *et al.*, 1998).

B: Immunoprecipitation of native X-ATM protein.

500 µg of extract were subjected to immunoprecipitation followed by Western blotting using the X-ATM antibody (lane 2). A 370 kDa polypeptide of the same size as that in the control extract (lane 1) is detected.

Figure 3.**A: Expression of X-ATM mRNA during development.**

RNAse protection assays using a 480bp probe from clone TBH4 of the X-ATM cDNA were performed using standard protocols at different stages of development (as indicated above the lanes) and from RNA prepared from the *Xenopus* XTC cell line (1st lane). The protected band is 480bp.

B: Expression of X-ATM protein during Development.

Equal amounts of protein extracts prepared from different stages were separated by electrophoresis followed by Western blot with the ATM affinity purified antibody. The protein is detected from stage 1 and the level of expression increases slightly through gastrulation (St. 13), and peaks at the time of formation of the neural tube and somites (St. 15-25), to slowly decrease thereafter. Drawings of the embryonic stages are shown above the autoradiogram.

Figure 4.**A: X-ATM during oogenesis.**

Manually dissected oocytes at different stages of vitellogenesis were collected. Equal amounts of protein were separated by electrophoresis and subjected to Western blotting. The stages of oogenesis are indicated below the lanes. The mobility was compared with that of X-ATM in a CSF extract (CSF).

B: Subcellular localization of ATM in the germline.

Protein samples from either complete stage VI oocytes (Total), stage VI oocyte cytoplasm (cytoplasm) or stage VI oocyte nuclei (nuclear) were processed for Western blotting. The two right lanes show a similar Western blot using either demembranated sperm nuclei or protein extracts prepared from nuclei.

Figure 5.**A: X-ATM expression throughout the meiotic cell cycle.**

Western blot analysis of oocytes progressing synchronously through meiosis was performed. $t=0$ is the time of addition of progesterone. GVBD₅₀ indicates the G2/M transition, the * indicates the interphase between metaphase of meiosis I and metaphase of meiosis II. X-ATM Western blot: top panel, Histone H1 kinase activity of the same samples : bottom panel.

B: X-ATM during the mitotic cell cycles

ATM levels during S phase and mitosis were compared by western blot analysis of "cycling extracts" prepared from *Xenopus* eggs. X-ATM levels were monitored by 6% SDS-PAGE and western blot analysis of extracts over a period of 100 minutes (top panel), corresponding to two cell cycles as determined by analysis of cdc2/cyclin B kinase activity (bottom panel). M phase, i.e. the kinase activity peaks, is indicated by *. Comparison of a mitotic extract (CSF), and an interphase extract (INT), also indicated a similar amount of ATM in these different cell cycle phases. For comparison, a HeLa whole-cell lysate was also analyzed on this gel. Human ATM antibody (Gately, D.P., Hittle J.C., Chan, G.K.T., and Yen, T.J. (1998) Mol. Biol. Cell. pp. 2361-2374) recognizes a band with a slightly faster mobility than *Xenopus* ATM.

Figure 6: X-ATM protein following DNA damage.

The level of ATM in extracts from control and γ -irradiated embryos between stage 8 and 10.5 was compared. Embryos were γ -irradiated (40 Gy) using a ⁶⁰Co source at stage 2. Embryos were collected and crushed at the indicated stages, and the soluble extract was analyzed by 6% SDS-PAGE followed by Western blot analysis. ATM in a mitotic extract (CSF) is also shown.

Figure 7: X-ATM complexes

200 μ g of egg extract were electrophoresed under native conditions and immunoblotted with X-ATM antibody (first lane). A band of approximately 450-500kD corresponding to the complex containing X-ATM is observed as indicated. The same sample was electrophoresed in the same polyacrylamide gradient gel under reducing and denaturing conditions (second lane) .The immunoreactive band migrates at 370kDa, the molecular weight of monomeric ATM.

Acknowledgments:

We would like to thank Dr. Yen for the gift of the human ATM antibody used in this study. This work is supported by U.S. Army grants DAMD 17-97-2-7075 to C.H. and DAMD 17-97-1-7071 and the Mallinkrodt Foundation to J.G.

REFERENCES

- Anderson, J.A., Lewellyn, A.L. & Maller, J.L. (1997). *Mol Biol Cell*, **8**, 1195-206.
- Athma, P., Rappaport, R. & Swift, M. (1996). *Cancer Genet Cytogenet*, **92**, 130-4.
- Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y. & Ziv, Y. (1998). *Science*, **281**, 1674-7.
- Barlow, C., Hirotsume, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J.N., Ried, T., Tagle, D. & Wynshaw-Boris, A. (1996). *Cell*, **86**, 159-71.
- Barlow, C., Liyanage, M., Moens, P.B., Tarsounas, M., Nagashima, K., Brown, K., Rottinghaus, S., Jackson, S.P., Tagle, D., Ried, T. & Wynshaw-Boris, A. (1998). *Development*, **125**, 4007-17.
- Baskaran, R., Wood, L.D., Whitaker, L.L., Canman, C.E., Morgan, S.E., Xu, Y., Barlow, C., Baltimore, D., Wynshaw-Boris, A., Kastan, M.B. & Wang, J.Y. (1997). *Nature*, **387**, 516-9.
- Beamish, H., Williams, R., Chen, P. & Lavin, M.F. (1996). *J Biol Chem*, **271**, 20486-93.
- Boder, E. & Sedgwick, R.P. (1970). *Psychiatr Neurol Med Psychol Beih*, **14**, 8-16.
- Brown, A.L., Lee, C.H., Schwarz, J.K., Mitiku, N., Piwnica-Worms, H. & Chung, J.H. (1999). *Proc Natl Acad Sci U S A*, **96**, 3745-3750.
- Brown, K.D., Ziv, Y., Sadanandan, S.N., Chessa, L., Collins, F.S., Shiloh, Y. & Tagle, D.A. (1997). *Proc Natl Acad Sci U S A*, **94**, 1840-5.
- Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B. & Siliciano, J.D. (1998). *Science*, **281**, 1677-9.
- Chen, G. & Lee, E. (1996). *J Biol Chem*, **271**, 33693-7.

- Chen, J., Birkholtz, G.G., Lindblom, P., Rubio, C. & Lindblom, A. (1998). *Cancer Res*, **58**, 1376-9.
- Chen, P., Gatei, M., O'Connell, M.J., Khanna, K.K., Bugg, S.J., Hogg, A., Scott, S.P., Hobson, K. & Lavin, M.F. (1999). *Oncogene*, **18**, 249-56.
- Concannon, P. & Gatti, R.A. (1997). *Hum Mutat*, **10**, 100-7.
- Cox, L.S., Midgley, C.A. & Lane, D.P. (1994). *Oncogene*, **9**, 2951-9.
- Dasso, M. & Newport, J.W. (1990). *Cell*, **61**, 811-23.
- Dreyer, C. & Hausen, P. (1983). *Dev Biol*, **100**, 412-25.
- Dumont, J.N. (1972). *J Morphol*, **136**, 153-79.
- Elson, A., Wang, Y., Daugherty, C.J., Morton, C.C., Zhou, F., Campos-Torres, J. & Leder, P. (1996). *Proc Natl Acad Sci U S A*, **93**, 13084-9.
- Ford, C.C. & Gurdon, J.B. (1977). *J Embryol Exp Morphol*, **37**, 203-9.
- Gately, D.P., Hittle, J.C., Chan, G.K.T. & Yen, T.J. (1998). *Mol Biol Cell*, **9**, 2361-74.
- Gautier, J. & Maller, J.L. (1991). *Embo J*, **10**, 177-82.
- Hendrickson, M., Madine, M., Dalton, S. & Gautier, J. (1996). *Proc Natl Acad Sci U S A*, **93**, 12223-8.
- Hensey, C. & Gautier, J. (1995). *Prog Cell Cycle Res*, **1**, 149-62.
- Hensey, C. & Gautier, J. (1997). *Mech Dev*, **69**, 183-95.
- Herzog, K.H., Chong, M.J., Kapsetaki, M., Morgan, J.I. & McKinnon, P.J. (1998). *Science*, **280**, 1089-91.
- Hoekstra, M.F. (1997). *Curr Opin Genet Dev*, **7**, 170-5.
- Khanna, K.K., Keating, K.E., Kozlov, S., Scott, S., Gatei, M., Hobson, K., Taya, Y., Gabrielli, B., Chan, D., Lees-Miller, S.P. & Lavin, M.F. (1998). *Nat Genet*, **20**, 398-400.
- Kumagai, A., Guo, Z., Emami, K.H., Wang, S.X. & Dunphy, W.G. (1998a). *J Cell Biol*, **142**, 1559-69.
- Kumagai, A., Yakowec, P.S. & Dunphy, W.G. (1998b). *Mol Biol Cell*, **9**, 345-54.
- Lavin, M.F. & Shiloh, Y. (1997). *Annu Rev Immunol*, **15**, 177-202.

- Lim, D.S., Kirsch, D.G., Canman, C.E., Ahn, J.H., Ziv, Y., Newman, L.S., Darnell, R.B., Shiloh, Y. & Kastan, M.B. (1998). *Proc Natl Acad Sci U S A*, **95**, 10146-51.
- Matsuoka, S., Huang, M. & Elledge, S.J. (1998). *Science*, **282**, 1893-7.
- Meyn, M.S. (1995). *Cancer Res*, **55**, 5991-6001.
- Morgan, S.E., Lovly, C., Pandita, T.K., Shiloh, Y. & Kastan, M.B. (1997). *Mol Cell Biol*, **17**, 2020-9.
- Murray, A.W. (1991). *Methods Cell Biol*, **36**, 581-605.
- Nakagawa, K., Taya, Y., Tamai, K. & Yamaizumi, M. (1999). *Mol Cell Biol*, **19**, 2828-34.
- Pudney, M., Varma, M.G. & Leake, C.J. (1973). *Experientia*, **29**, 466-7.
- Sagata, N., Oskarsson, M., Copeland, T., Brumbaugh, J. & Vande Woude, G.F. (1988). *Nature*, **335**, 519-25.
- Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D.A., Smith, S., Uziel, T., Sfez, S. & et al. (1995). *Science*, **268**, 1749-53.
- Scott, S.P., Zhang, N., Khanna, K.K., Khromykh, A., Hobson, K., Watters, D. & Lavin, M.F. (1998). *Biochem Biophys Res Commun*, **245**, 144-8.
- Shafman, T., Khanna, K.K., Kedar, P., Spring, K., Kozlov, S., Yen, T., Hobson, K., Gatei, M., Zhang, N., Watters, D., Egerton, M., Shiloh, Y., Kharbanda, S., Kufe, D. & Lavin, M.F. (1997). *Nature*, **387**, 520-3.
- Shiloh, Y. (1998). *Biochim Biophys Acta*, **1378**, R11-8.
- Smilenov, L.B., Morgan, S.E., Mellado, W., Sawant, S.G., Kastan, M.B. & Pandita, T.K. (1997). *Oncogene*, **15**, 2659-65.
- Soussi, T., Caron de Fromental, C., Mechali, M., May, P. & Kress, M. (1987). *Oncogene*, **1**, 71-8.
- Stankovic, T., Weber, P., Stewart, G., Bedenham, T., Murray, J., Byrd, P.J., Moss, P.A. & Taylor, A.M. (1999). *Lancet*, **353**, 26-9.
- Swift, M., Chase, C.L. & Morrell, D. (1990). *Cancer Genet Cytogenet*, **46**, 21-7.
- Taylor, A.M. (1998). *Int J Radiat Biol*, **73**, 365-71.

- Tchang, F., Gusse, M., Soussi, T. & Mechali, M. (1993). *Dev Biol*, **159**, 163-72.
- Vaziri, H., West, M.D., Allsopp, R.C., Davison, T.S., Wu, Y.S., Arrowsmith, C.H., Poirier, G.G. & Benchimol, S. (1997). *Embo J*, **16**, 6018-33.
- Walter, J., Sun, L. & Newport, J. (1998). *Mol Cell*, **1**, 519-29.
- Wang, Y., Farmer, G., Soussi, T. & Prives, C. (1995). *Oncogene*, **10**, 779-84.
- Waterman, M.J., Stavridi, E.S., Waterman, J.L. & Halazonetis, T.D. (1998). *Nat Genet*, **19**, 175-8.
- Xu, Y., Ashley, T., Brainerd, E.E., Bronson, R.T., Meyn, M.S. & Baltimore, D. (1996). *Genes Dev*, **10**, 2411-22.
- Xu, Y. & Baltimore, D. (1996). *Genes Dev*, **10**, 2401-10.
- Yuille, M.A. & Coignet, L.J. (1998). *Recent Results Cancer Res*, **154**, 156-73.

Figure 1A

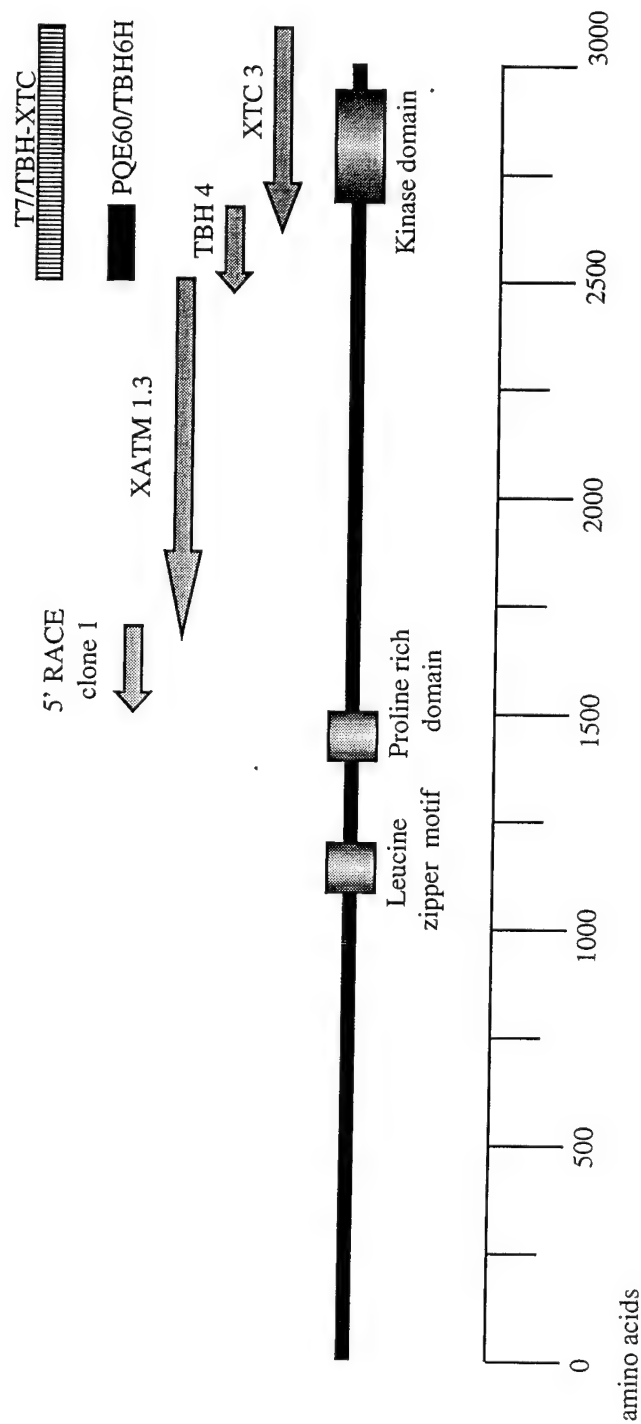


Figure 1B

X-ATM	DVSTRSLLLC	LDLHVRVCA	AVTHCEDALE	CHHHVIVGSL	40	X-ATM	MFOIKQYNPS	QLGVSEWQLE	EAQIFWDKEE	PSLAREILKQ	840
H-ATM	DVSLRSFSLC	CDLLSQVCQT	AVTYCKDALE	NHLHVIVGTL	1520	H-ATM	IFQIKQYNSV	SCGVSEWQLE	EAQVFWAKKE	QSLALSILKQ	2310
Consensus	DVS..RS..LC	.DLL..VC..	AVT..C..DALE	.N.HVIVG..L		Consensus	.FQIKQYN..	..GVSEWQLE	EAQ..FW..K..E	.SLA...ILKQ	
X-ATM	IPLANSQESI	QEKVCDLLNF	LVLENKDNEN	LYHTIKLLDP	80	X-ATM	MINKLEIKSF	EVENDSRRLR	LYAESLRLCG	KWLAETCLES	880
H-ATM	IPLYVEQVEV	QKQVLDLLKY	LVIDNKNEN	LYITIKLLDP	1560	H-ATM	MIKKLDASC-	-AANNPSLKL	TYTECLRVCG	NWLAETCLEN	2348
Consensus	IPL...Q...Q	Q..V..DLL..	LVI..NKNEN	LY..TIRLLDP		Consensus	MI..KL.....	...N...L..L	.Y..E..LR..CG	.WLAETCLE..	
X-ATM	FPDIPLFKNL	ROAQHKIKYS	KGPFSLIKEI	QNFLSVSVCD	120	X-ATM	PTVIMQNYLE	KAVEFAG-YS	DGAGEKLQEG	RMKAPLSLAR	919
H-ATM	FPDHVFKDL	RITQKQIKYS	RGPFSLLEEI	NHFLSVSVVD	1600	H-ATM	PAVIMQTYLE	KAVEVAGNYD	GESSDELRLG	KMKAPLSLAR	2388
Consensus	FPD...PK..L	R...QKIKYS	.GPFSL..EI	..FLSVSV..D		Consensus	P..VIMQ..YLE	KAVE..AG..Y..L...G	.HKAFLSLAR	
X-ATM	SLPLTRLEGL	NDLRKQLEQH	KDQIKELVRD	CQCTPQDSVI	160	X-ATM	FSDAQYQRID	NYMKSSEFEN	KQALLRKAKE	EVGLIKQHKV	959
H-ATM	ALPLTRLEGL	KDLRRQLELH	KDQVMDVIRA	SQDNPDQDGI	1640	H-ATM	FSDTQYQRIE	NYMKSSEFEN	KQALLRKAKE	EVGLIREHKI	2428
Consensus	.LPLTRLEGL	.DLR..QLE..H	KDQ.....R	..Q...PQD...		Consensus	FSD..QYQRI..	NYMKSSEFEN	KQALL..AKE	EVGL...HK..	
X-ATM	ASLVVNLLQL	SKNAVHQSG	KEVLEAVGSC	LGEGLPIDFS	200	X-ATM	QNNRYTVKVE	RELQDLDECAI	LALREDRKRK	LCKAVENYIS	999
H-ATM	VKLIVNLLQL	SKNAVHTGE	KEVLEAVGSC	LGEVGPIDFS	1680	H-ATM	QTNRYTVKVQ	RELELDELAL	RALKEDRKRK	LCKAVENYIN	2468
Consensus	..LVVNLLQL	SK.A.....	KEVLEAVGSC	LGE..GPIDFS		Consensus	Q..NRYTVKV..	REL..LDE..A..	.AL..EDRKRK	LCKAVENYI..	
X-ATM	NIALQHQHKD	SVYLKADKVF	EKEELQCVLV	MLTLINNALT	240	X-ATM	CLVSGEEHDM	WIFRLCSLWL	ENSAVDVNS	MMRQDAQKIP	1039
H-ATM	TIATQHSKDA	S-YTKALKLF	EDKELQWTFI	MLTYLNNTLV	1719	H-ATM	CLLSGEEHDM	WVFRCLSLWL	ENSGVSEVNG	MMKRGDKMIP	2508
Consensus	.IA.Q...K...S	.Y..KA..K..F	E..KELQ....	MLT...NN..L.		Consensus	CL..SGEEHDM	W..FRCLSLWL	ENS..VS..VN..	MM...D...KIP	
X-ATM	DHCIOVRSAA	ATCLKNILAT	KTGAMFEAC	KDKGEMPLY	280	X-ATM	SHKFLPLMYQ	LAARMGTMM	GNPGFHDVLN	NLIGRISMDH	1079
H-ATM	EDCVKVRSA	ATCLKNILAT	KTGHSFWEIY	KMTTDPMLY	1759	H-ATM	TYKFLPLMYQ	LAARMGTMM	GGLGPFHVLN	NLISRISMDH	2548
Consensus	..C...VRSAA	.TCLKNILAT	KTG...FWE..	K...PML..Y		Consensus	..KFLPLMYQ	LAARMGT..M	G...GFH..VLN	NLI..RISMDH	
X-ATM	LQFPFRPKKK	FLEVEEIORE	GALESDDPG	IWIPONENHE	320	X-ATM	PHHTLFIIILA	LANANKDDQL	MKAFAVKRSR	LTKNAPKQIS	1119
H-ATM	LQFPFRSRKK	FLEVPRFDKE	NPFEGLDDIN	LWIPLSENHD	1799	H-ATM	PHHTLFIIILA	LANANRDEFL	TKPEVARRSR	ITKNVPKQSS	2588
Consensus	LQFPFR...KK	FLEV.....E	...E.LDD..	.WIP...ENH..		Consensus	PHHTLFIIILA	LANAN..D...L	.K.E...RSR	.TKN..PKQ..S	
X-ATM	SWIKHLTCTL	LESQGVKSEV	LLLLKPMCEV	KADFCQAVVP	360	X-ATM	QLDKERMEAA	RHIVDTIKKR	RTDMVRDVER	LCDAYITLAN	1159
H-ATM	IWKITLTCAF	LDSGGTKCEI	LQLLKPMCEV	KTDQCQTVLP	1839	H-ATM	QLDEDRTTAA	NRIICTIRSR	RPQMVRSVEA	LCDAYIILAN	2628
Consensus	.WIK..LTC...L	.SGG..K.E..L	.LLKPMCEV	K..DFCQ..V..P		Consensus	QLD...R.EAA	..I...TI...R	R...MVR..VE..	LCDAIYI..LAN	
X-ATM	YIVHNILLND	SNQWTWRTLS	KNVQFFFTSC	CRSLPSSRS	400	X-ATM	MDANQWKSQR	NAIPIPSDQP	ITKLNLKHDV	VIPTMEIKVD	1199
H-ATM	YLHIDILLQD	TNESNRNLLS	LRHFSQTSRS		1879	H-ATM	LDATQWKTQR	KGINIPADQP	ITKLKNEEDV	VVPTMEIKVD	2668
Consensus	Y...H..ILL..D	.N...WR..LLS	..VQ..FPTSC	.R.....SRS		Consensus	.DA.QWK..QR	..I..IP..DQP	ITKL...L..DV	V..PTMEIKVD	
X-ATM	ATPASSDES	EGVARGAVDI	ASRRTMLTMV	EHLRQRRPV	440	X-ATM	PSGEYENLVT	IVSFKPEFRL	AGGLNLPKII	DCVSGDGKER	1239
H-ATM	TTPANLDS	EHFPRCCLDK	KSQRTMLAVV	DYMRQRKRP	1919	H-ATM	HTGEYGNLVT	IQSFKAERFL	AGGVNLPKII	DCVSGDGKER	2708
Consensus	.TPA...DSES	E...R...D..D	.S.RTML..V	...R.Q..RP..		Consensus	..GEY..NLVT	I..SFK..EFRL	AGG..NLPKII	DCVSGDGKER	
X-ATM	SGTAFDDNF	LDLNYLEVAM	AVQSCAAHFT	ALLYSEIYTD	480	X-ATM	RQLVKGDDL	QDAVMQVVF	QMCNTLLQRN	SETRKRRLTI	1279
H-ATM	SGTIFNDAF	LDLNYLEVAK	VAQSCAAHFT	ALLYAEIYAD	1959	H-ATM	RQLVKGDDL	QDAVMQVVF	QMCNTLLQRN	SETRKRRLTI	2748
Consensus	SGT..F..D.FW	LDLNYLEVA..	..QSCAAHFT	ALLY..EY..D		Consensus	RQLVKG..DDL	QDAVMQVVF	QMCNTLLQRN	..SETRKRRLTI	
X-ATM	KVKQDGEQRT	SANRSNARKC	LKFEQGSQTL	DITGLSEKSK	520	X-ATM	RRYKVPLSH	RSQVLEWCTG	TVPIGEVLN	DKDGAHKRYR	1319
H-ATM	KKSMDQGE--	-----KRS	LAFEQGSQST	TISSLSEKSK	1990	H-ATM	CTYKVPLSQ	RSQVLEWCTG	TVPIGEVLN	NEDGAHKRYR	2788
Consensus	K...D.....L.FEQGSQ..	.I..LSEKSK			Consensus	..YKVPLS..	RSQVLEWCTG	TVPIGE..LVN	..DGAHKRYR	
X-ATM	EETGISLQDL	LMDIYRSIGE	PDSLYGCGGG	KMLHPLARIR	560	X-ATM	PGDYGSLLCQ	RKMMEVQRGR	FEKEYQMFNL	VCENFRPVFR	1359
H-ATM	EETGISLQDL	LLEIYRSIGE	PDSLYGCGGG	KMLQPIRLR	2030	H-ATM	PNDFAFQCC	KMMEVQKKS	FEKEYVFMD	VCONFPVFR	2828
Consensus	EETGISLQDL	L...IYRSIGE	PDSLYGCGGG	KML.P...R..R		Consensus	P..D....QCC	.KMMEVQ...K	FEKEY..F...VC	..NF..PVFR	
X-ATM	TYEHEAKWKG	ALVTFDLEMN	LPPVTRQAGI	MQALQNLGLC	600	X-ATM	YFCMEKFLDP	AMWFEKRLAY	TRSVATSSIV	GYIVGLGDRH	1399
H-ATM	TYEHEAMWKG	ALVTYDLETA	IPSSSTRQAGI	IQALQNLGLC	2070	H-ATM	YFCMEKFLDP	AIWFEKRLAY	TRSVATSSIV	GYILGLGDRH	2868
Consensus	TYEHEA.WKG	ALVT..DLE..	.P..TRQAGI	.QALQNLGLC		Consensus	YFCMEKFLDP	A..WFEKRLAY	TRSVATSSIV	GYI..GLGDRH	
X-ATM	HMLSTYLRGL	EHENAESWSE	LQEIHFQAAW	RNMQWDDSLP	640	X-ATM	VQNILIDEES	AELVHIDLGV	APEQGGKILPT	PETVPFRLTR	1439
H-ATM	HILSVYLKGL	DYENKDWCE	LEELHYQAAW	RNMQWDHCTS	2110	H-ATM	VQNILINEQS	AELVHIDLGV	APEQGGKILPT	PETVPFRLTR	2908
Consensus	H..LS..YL..GL	..EN..W..E..L	E..H..Y.QAAW	RNMQWDHCTS		Consensus	VQNILI..E..S	AELVHIDLGV	APEQGGKILPT	PETVPFRLTR	
X-ATM	TKNETSGPGY	HESLYRAVQS	LRDKEFCGFH	DHIKYARVKE	680	X-ATM	DIVDGMGITG	VEGVFRRCCE	KTMEVMNRNQ	EALLTIVEVL	1479
H-ATM	VSKEVEGTSY	HESLYNALQS	LRDREFSTFY	ESLYARVKE	2150	H-ATM	DIVDGMGITG	VEGVFRRCCE	KTMEVMNRNQ	ETLLTIVEVL	2948
Consensus	...E..G...Y	HESLY..A..QS	LRD..EF...F..	...KYARVKE		Consensus	DIVDGMGITG	VEGVFRRCCE	KTMEVMNR..Q	E..LLTIVEVL	
X-ATM	VEELCSGSLE	SVYSLYPALC	RLQAIGELAS	VGMFMSRSIT	720	X-ATM	LYDPLFDWTM	NPLKALYLQ	--DEVDLNA	TLGGDDPECN	1516
H-ATM	VEEMCKRSLE	SVYSLYPTLS	RLQAIGELAS	IGELFMSRSVT	2190	H-ATM	LYDPLFDWTM	NPLKALYLQ	RPEDETELHP	TLNADDQCEK	2988
Consensus	VEE.C...SLE	SVYSLYP..L..	RLQAIGEL..S	.G...FSRS..T		Consensus	LYDPLFDWTM	NPLKALYLQ	...DE..L...TL	..DD..EC..	
X-ATM	DDGLKDTFLK	WQSQQLLKD	SDFEFLEPVL	SLRSVILETL	760	X-ATM	RNSCD-SQSV	NKVAERVLRL	LQEKLTGVEE	GMVLSVGGQV	1555
H-ATM	HRQLSEVYIK	WQKHSQLLK	SDFSQFQPMI	ALRTVILEIL	2230	H-ATM	RNLSDDIDQS	DKVAERVLRL	LQEKLTGVEE	QTVLSVGGQV	3028
Consensus	...L.....K	WQ...SQLLKD	SDF..F..EP..	..LR..VILE..L		Consensus	RN...D...QS..	.KVAERVL..R	LQEKL..GVEE	G..VLSVGGQV	
X-ATM	LQEEKKQPSQ	ESLKDILTKH	LLDLSRIARS	AGNTOLPEKA	800	X-ATM	NHLIQQAMPD	KNLSRLFPGW	KAWVZ	1580	
H-ATM	MEKEMDSQR	ECIKDILTKH	LVELSLIART	FKNTOLPERA	2270	H-ATM	NLLIQQAIDP	KNLSRLFPGW	KAWV	3052	
Consensus	...E.....E	..KDILTKH	L..LS..AR..	..NTOLPE..A		Consensus	N..LIQQA..DP	KNLSRLFPGW	KAWV..		

Figure 2

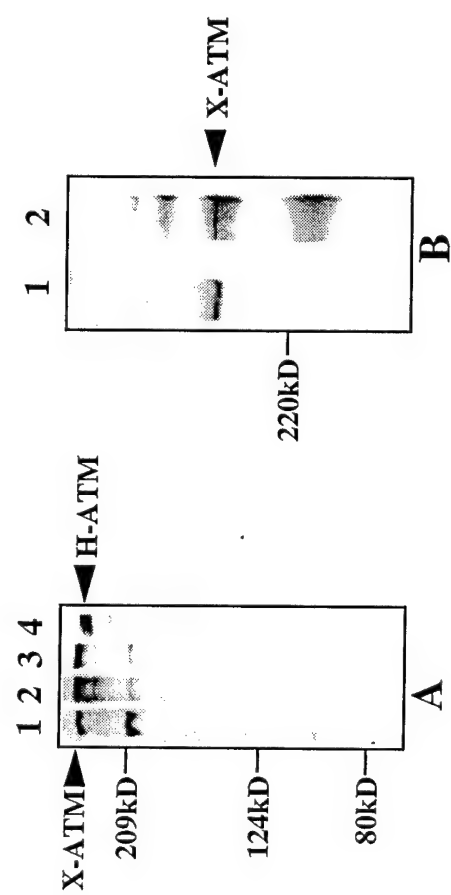


Figure 3

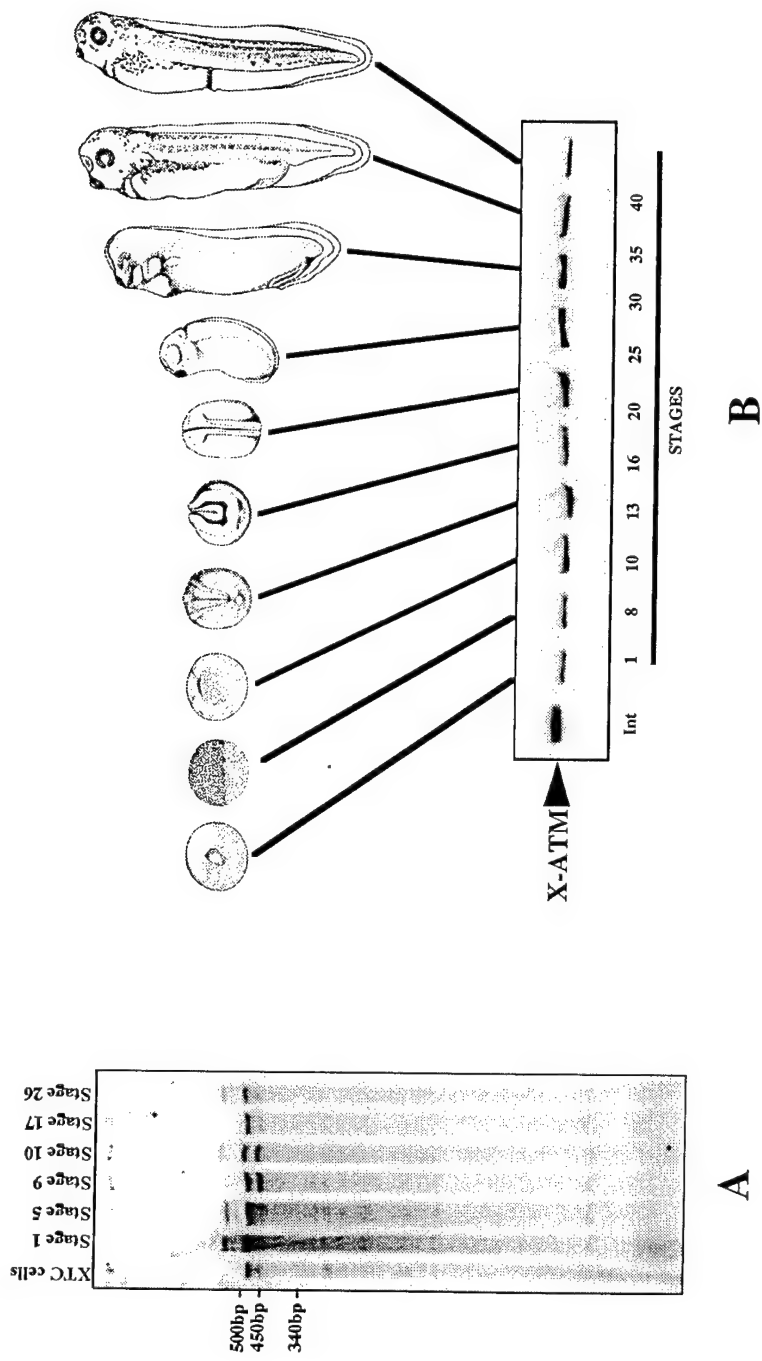


Figure 4

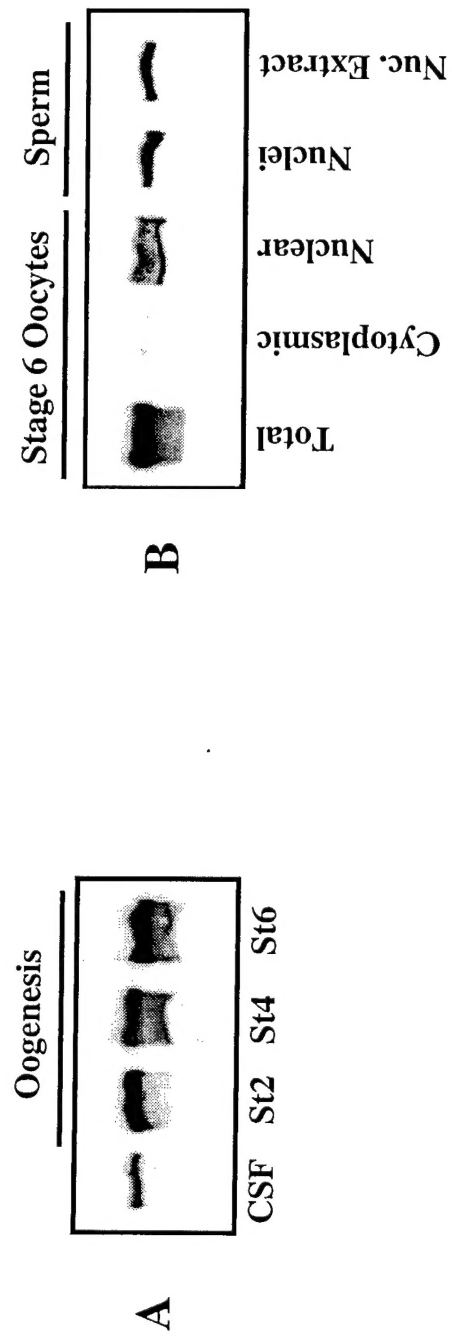


Figure 5

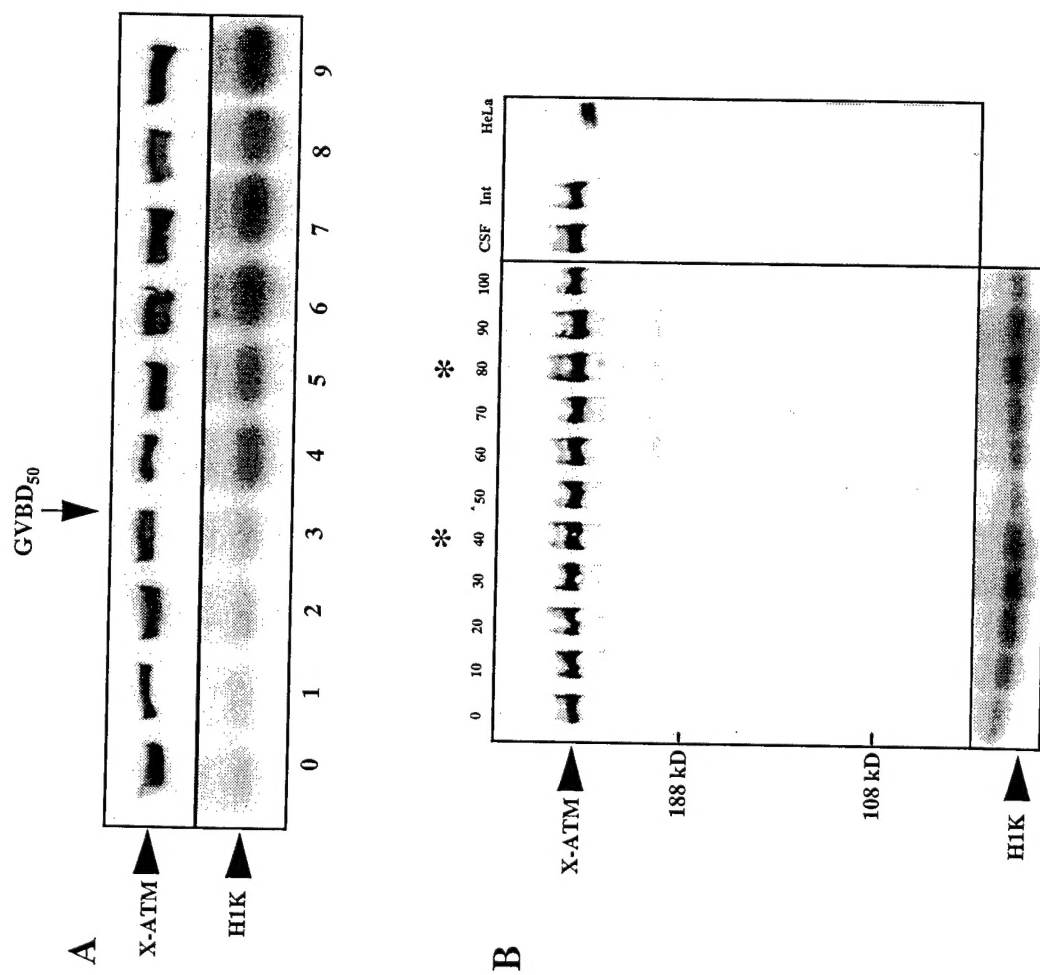


Figure 6

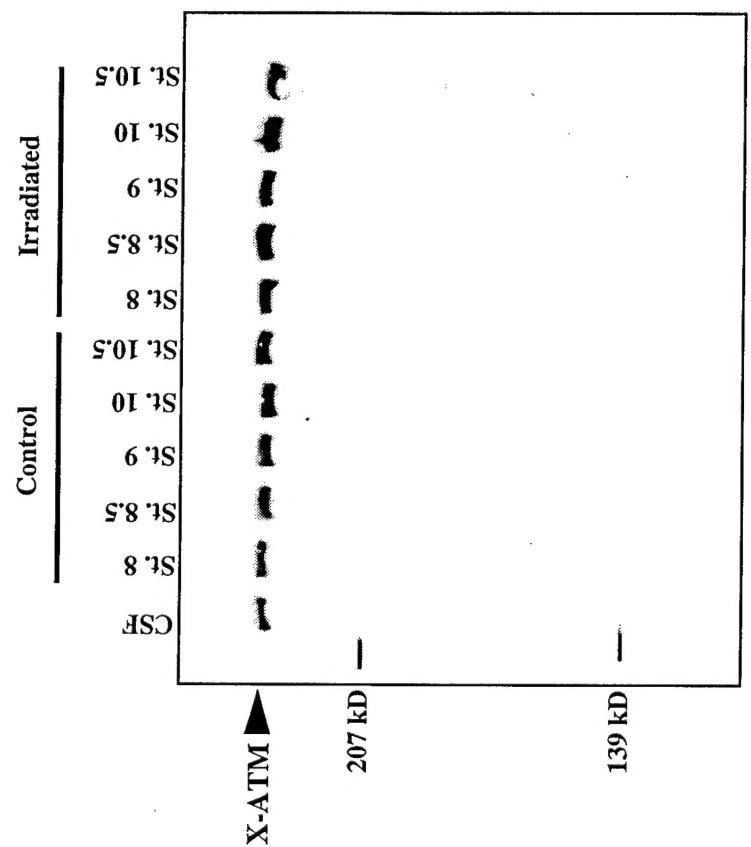


Figure 7

